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A Correlation between Mutation of the p53 Gene and Histological Heterogeneity in Differentiated Adenocarcinomas of the Lung, with Reference to Stepwise Progression and Metastatic Ability

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ABSTRACT

I examined 15 cases of differentiated adenocarcinoma of the lung which showed mutation of the p53 gene and whose primary lesions could be divided into three components (peripheral component, intermediate component and central component). Mutation of the p53 gene in each component was examined by an immunohistochemical method and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis in order to elucidate the correlation between histological types in the adenocarcinoma and mutation of the p53 gene. Furthermore, immunohistochemical detection of Ki-67 antigen and the ploidy pattern were examined for each component of the primary lesions and metastatic lesions. As a result, mutation of the p53 gene was detected mainly in the intermediate and central components, that is, in 13 cases in the intermediate component and in all cases in the central component. Higher proliferative activity as well as a DNA aneuploidy pattern were seen in these areas. In contrast, the peripheral component showed mutation of the p53 gene in only 4 cases. Lower proliferative activity and a normal diploidy pattern were seen in this area. As for the metastatic lesions, mutations of the p53 gene were detected in the metastasis of the lymph nodes and brain, as well as in the intermediate and central components of the primary lesions. Moreover, these metastatic lesions showed a higher proliferative activity which was similar index to the central or intermediate one. In 2 cases the metastasis of the lymph node showed an aneuploidy pattern as well as the intermediate and central components. On the other hand, in two metastatic lung tumors no mutation of the p53 gene was detected and lower proliferative activity was seen as observed in the peripheral one. From these results, I conclude that progression of adenocarcinoma of the lung occurs in the intermediate and central components, and is closely related to mutation of the p53 gene.

Key words: *p53 gene, Lung adenocarcinoma, Histological heterogeneity*

Recently it has been proposed that the process of "multistep carcinogenesis", namely initiation, promotion and progression, is associated with a stepwise accumulation of genetic abnormalities and is essentially the natural course of development of various types of carcinoma. For example, it is well known that colorectal carcinoma carry several abnormalities of the oncogenes, tumor suppressor genes and chromosome regions including APC, ras, MMC, p53, DCC and chromosome 17 or 22, and that these abnormalities accumulate during the course of the "adenoma-carcinoma sequence"[2]. However, our knowledge of the course of carcinogenesis in lung carcinoma is very limited.

Well or moderately-differentiated adenocarcinoma of the lung often show histological heterogeneity within one tumor. That is, a papillary structure with thin stroma is noted in the peripheral region and a papillary structure with thick stroma in the intermediate region, while solid or tubular structures with abundant fibrous stroma exist in the central region. On the basis of this histological heterogeneity within one tumor, Nomori et al showed by means of DNA-cytofluorometrical analysis that the intermediate component with thick stroma metastasizes to distant organs, while the peripheral component with thin stroma hardly metastasizes at all[9]. By morphometrical and flow cytometrical DNA analysis on 5 well-differentiated adenocarcinomas of the lung we speculated that the adenocarcinoma cells in the intermediate component with thick stroma resembled those in the metastasis of the mediastinal lymph nodes and brain, while those in the peripheral component with thin stroma formed intrapulmonary metastasis[20]. From these results, it appears that the histological heterogene-

ity in the differentiated adenocarcinomas of the lung correlates to their malignant or metastatic potential and that the progressive process of carcinoma can be detected among the primary tumor. Furthermore, it has been reported that mutation of the p53 gene is frequently seen in adenocarcinoma of the lung[4,6,7], but its significance or role in the process of carcinogenesis and/or progression is still unknown. It is, therefore, interesting to examine the correlation between histological types in the adenocarcinoma of the lung and mutation of the p53 gene. In the present study, an immunohistochemical method and a polymerase chain reaction-denaturing gradient gel electro-phoresis (PCR-DGGE) analysis were utilized for the detection of p53 gene mutation in the three components of the primary tumor and the metastatic lesions. Analysis of the DNA pattern and immunohistochemical observation of Ki-67, one of the markers of growth fraction, were also performed in each component in order to elucidate the correlation between abnormality of the p53 gene and malignant potential.

MATERIALS AND METHODS

Materials: From our surgical file of lung adenocarcinoma, surgically resected at Hiroshima University Hospital during the period 1980 to 1992, 15 cases with mutation of the p53 gene were selected for the present study. In these cases, the primary tumor could be histologically divided into three components: the peripheral component, showing a papillary pattern with thin stroma (Fig. 1a), the intermediate component, with a papillary pattern with thick stroma (Fig. 1b), and the central component, with a solid or tubular pattern with abundant fibrous stroma (Fig. 1c).

The fifteen cases comprised 7 males and 8 females. The patients' ages ranged from 43 to 75 years old with an average of 65 (Table 1). The assignments to T, N, M categories and pathological stages were determined according to the General Rule for Clinical and Pathological Record of Lung Cancer (1987)[17]. Eight cases had metastasis at the time of surgery, as shown in Table 1, and these metastatic tumors were also analysed.

Immunohistochemistry: Histological sections from formalin-fixed and paraffin-embedded tissue were prepared, and microwave-oven processing, which is known to activate the antigen, was performed according to Cattoretti's method[1].

The antibody DO-7 (Novocastra Laboratories Ltd., Newcastle, England) was a mouse monoclonal antibody, which reacts with mutant form as well as the wild form of p53 protein[19]. The antibody MIB-1 (Immunotech, Marseille, France), was a mouse monoclonal antibody against recombinant parts of Ki-67 antigen[1].

Immunoperoxidase staining was performed using the Streptoavidin-Biotin method, as follows: after the blocking of endogenous peroxidase activity with 0.3% H_2O_2 in absolute methanol and blocking of nonspecific binding sites with normal serum, the sections were incubated with the antibodies for 18 hrs at 4°C. The immunoreactive compounds were detected by Stravigen BSA-PO Kit (BioGenex laboratories, San Ramon, U.S.A.) with diaminobenzidine (Sigma, ST. Louis, U.S.A.)

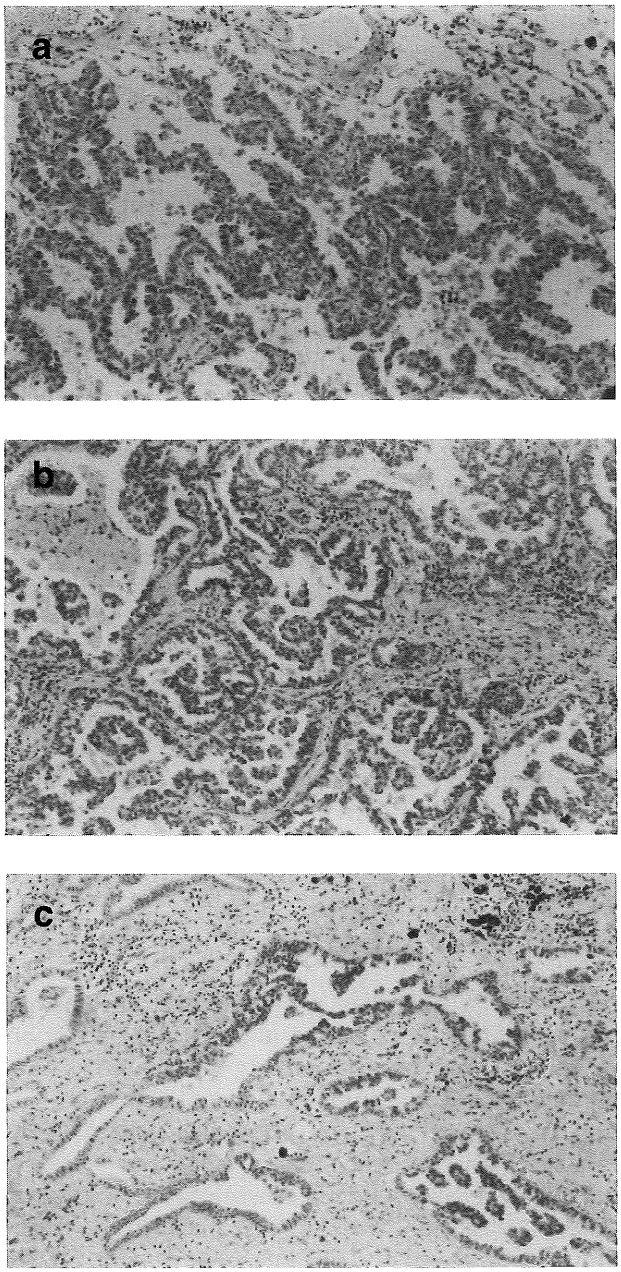

Fig. 1. (a) Peripheral region of case no.6. Carcinoma showed a papillary structure with thin stroma (H&E stain, ×60).
(b) Intermediate region of case no.6. Carcinoma showed a papillary structure with thick stroma (H&E stain, ×60).
(c) Central region of case no.6. Carcinoma showed a tubular structure with desmoplastic reaction (H&E stain, ×60).

Table 1. Well differentiated adenocarcinomas examined in this study

Case no.	Age	Sex	Stage	(TNM)	Metastatic site		
					LN	Lung	Brain
1	52	F	I	(100)			
2	65	F	I	(100)			
3	74	M	I	(100)			
4	58	M	I	(100)			
5	66	F	I	(100)			
6	74	M	IIIa	(120)	●		
7	73	M	IIIa	(120)	●		
8	74	M	IV	(101)		●	
9	43	M	IV	(122)	●		●
10	75	M	IIIa	(320)	●		
11	54	F	IV	(421)	●	●	
12	61	F	IIIa	(120)	●		
13	66	F	I	(200)			
14	53	F	I	(200)			
15	58	F	IIIa	(220)	●		

as a chromogen. The counterstaining of nuclei was done with methylgreen.

The immunoreactivity to DO-7 was evaluated as follows: grade 4, the population of positive cells among all of the carcinoma cells was more than 75%; grade 3, the population ranged from 50 to 74%; grade 2, the population ranged from 25 to 49%; grade 1, the population was less than 25%; grade 0, no positive cells were present. The labeling index (L.I.) for Ki-67 was determined by observation of more than 1000 carcinoma cells. These evaluations were performed independently on each of the three components within the primary tumor.

Flow cytometrical analysis on DNA: Three 50μm-thick sections from paraffin-embedded tumor tissues were cut, and sections from each of the three components were separately prepared. Preparation of the cell suspensions and measurement of nuclear fluorescence were performed according to the method reported by Vindel et al[18].

PCR-DGGE analysis on the p53 gene: PCR-DGGE analysis was performed to confirm the mutation of the p53 gene in the regions between exon 5 through 9. The oligodeoxynucleotide primers for PCR used in the present study were synthesized according to a previous report[5] with slight modifications (Fig. 2). Genomic DNA prepared from paraffin samples of each component was subjected to amplification at each exon including the exon-intron boundary, then the PCR products were analysed by the DGGE method, described by Takahashi et al[16]. Namely, parallel gradient gel electrophoresis was performed using apparatus (Model SE-520, Vertical Flat Gel). The gel, measuring 20 cm × 20 cm × 0.5mm thick, consisted of a 6.5% polyacrylamide gel in TAE buffer

Exon 5	5' - TGGGCAACCAGCCCTGCTGT - 3'
	5' - TGCCCTGACTTTCAACTCTG - 3'
Exon 6	5' - TGGTTGCCCAGGGTCCCCAG - 3'
	5' - GGAGGGCCACGGACAACCA - 3'
Exon 7	5' - CTTGCCACAGGTCTCCCCAA - 3'
	5' - AGGGGTCAGCGGCAAGCAGA - 3'
Exon 8-9	5' - TTGGGAGTAGGAGCGGCCT - 3'
	5' - AGTGTTAGACTGGAAACTTT - 3'

Fig. 2. PCR primers used in this study.

(40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, adjusted with acetic acid to pH 7.4). It was prepared 2 cm up from the bottom of the gel to prevent leakage of the denaturant from the gel into the electrode solution during electrophoresis. The gel was composed of a fixed acrylamide concentration [acrylamide in gel = 6.5% (wt/vol); bisacrylamide in total acrylamide = 2.6% (wt/wt)] in TAE buffer with a linearly increasing concentration gradient of the denaturant. In most of the experiments, gels with a denaturant gradient of 0–100% were used (100% denaturant = 7 M urea and 40% formamide). The electrophoresis chambers were maintained at 60°C with a circulating heater and the parallel gel was run at 200 V for 17 hrs.

RESULTS

Immunohistochemical findings of p53 (Table 2):

The immunohistochemical finding of DO-7 in case no. 6 is shown in Fig. 3. In the peripheral

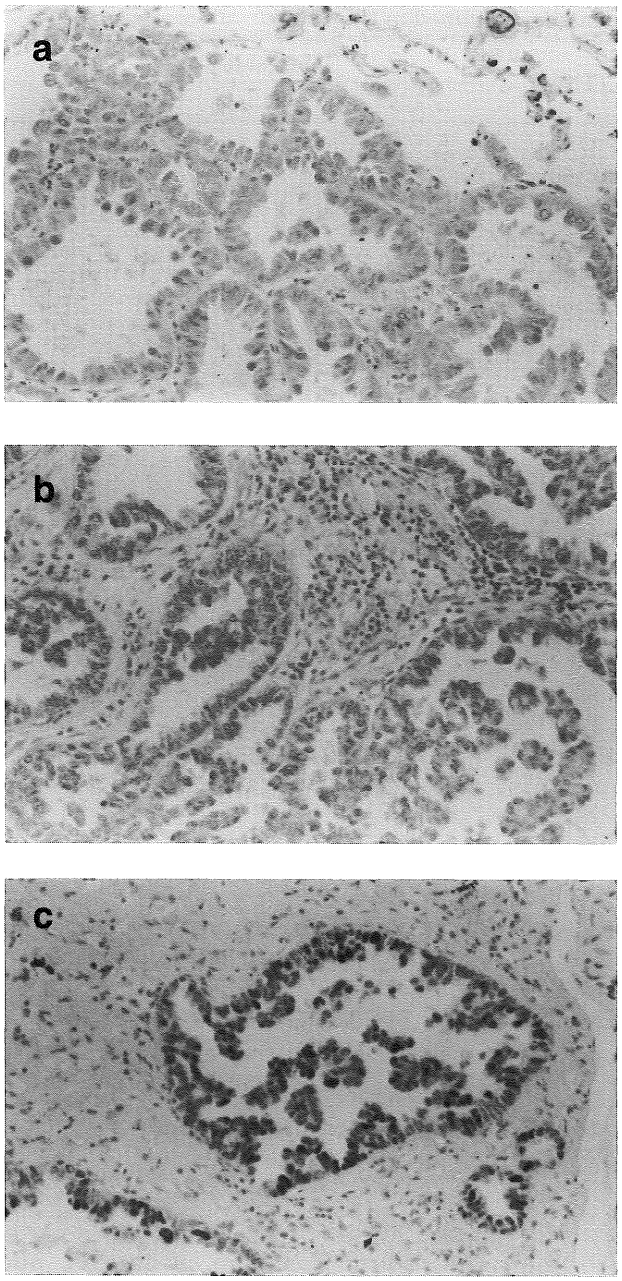

Fig. 3. (a) Peripheral component of case no.6. None of the carcinoma cells showed immunoreactivity of DO-7 (SAB method, ×200).
(b) Intermediate component of case no.6. Few carcinoma cells showed immunoreactivity of DO-7 (SAB method, ×200).
(c) Central component of case no.6. Many carcinoma cells, at least more than 50%, showed immunoreactivity of DO-7 (SAB method, ×200).

and intermediate components of the primary tumor, very few carcinoma cells showed immunoreactivity to DO-7(Fig. 3a, b), while in the central component and metastatic tumor in the lymph node, more than 50% of the carcinoma cells were

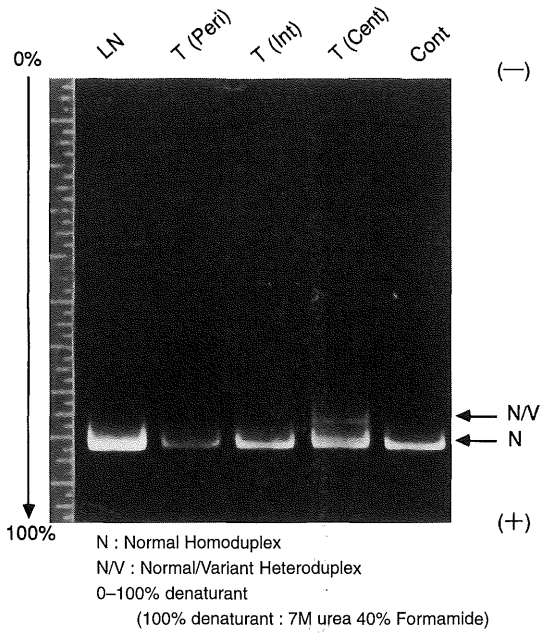

Fig. 4. PCR-DGGE (p53: exon6): Heteroduplex between normal and mutant sequences is noted in addition to a normal homoduplex band in the central component. Other components of primary tumor and metastatic lymph node exhibit only a single band corresponding to the normal homoduplex.

positive (Fig. 3c). A summary of the immunoreactivity to DO-7 in each of the components is shown in Table 2. The immunoreactivity to DO-7 exhibited a gradual decrease from the central component to the peripheral component. Compared with the primary tumor, the immunoreactivity to DO-7 in the metastasis of the lymph nodes and brain was similar to that in the central and intermediate components, while the lung metastasis showed a lower grade similar to that of the peripheral component.

PCR-DGGE analysis of the p53 gene (Table 2):

PCR-DGGE analysis was performed on all cases except for case no. 3. The result of exon 6 in case no. 6 is shown in Fig. 4. A heteroduplex band between normal and mutant sequences was noted in addition to a normal homoduplex band in the central component of the primary tumor. Other components of the primary tumor and metastatic tumor exhibited only a single band, corresponding to the normal homoduplex. These findings indicate that mutation of exon 6 occurred only in the central component. However, mutation could not be detected in the metastasis of the lymph node in spite of a positive immunoreactivity to DO-7. The reason for this discrepancy could be that the population of mutated carcinoma cells was smaller than that of normal lymphocytes in the lymph node.

The correlation between the immunohistochemical findings to DO-7 and PCR-DGGE analysis is

Table 2. Immunohistochemical observation of p53 oncoprotein and PCR-DGGE analysis of mutation of the p53 gene in primary tumors and metastatic lesions

Case no.	Primary tumor						Metastatic site					
	P		I		C		LN		Lung		Brain	
	DO7	(DGGE)	DO7	(DGGE)	DO7	(DGGE)	DO7	(DGGE)	DO7	(DGGE)	DO7	(DGGE)
1	2	(NI)	4	(E6)	4	(E6)						
2	0	(NI)	0	(NI)	4	(E6)						
3	1	(NE)	2	(NE)	3	(NE)						
4	2	(E6)	2	(E6)	2	(E6)						
5	1	(E7)	3	(E7)	4	(E7)						
6	0	(NI)	1	(NI)	3	(E6)	3	(NI)				
7	1	(NI)	3	(E8–9)	2	(E8–9)	4	(NI)				
8	0	(NI)	0	(NI)	2	(E5)			0	(NI)		
9	1	(NI)	1	(NI)	3	(E6)	3	(E6)			3	(E6)
10	1	(NI)	2	(NI)	4	(E6)	3	(E6)				
11	1	(NI)	3	(E5)	2	(E5)	2	(E5)	1	(NI)		
12	1	(NI)	4	(E7)	3	(E7)	4	(E7)				
13	2	(E6)	4	(E6)	4	(E6)						
14	3	(E8–9)	3	(E8–9)	3	(E8–9)						
15	1	(NI)	3	(E5)	4	(E6)	4	(NI)				

Grade 0: none
Grade 1: 0< <25
Grade 2: 25≦ <50
Grade 3: 50≦ <75
Grade 4: 75≦

P: peripheral component
I: intermediate component
C: central component
NI: not indicated for mutation
NE: not examined
E: exon

Table 3. Immunohistochemical observation of Ki-67 and DNA ploidy pattern in primary tumors and metastatic lesions

Case no.	Primary tumor						Metastatic site					
	P		I		C		LN		Lung		Brain	
	L.I.	DNA(D.I.)	L.I.	DNA(D.I.)	L.I.	DNA(D.I.)	L.I.	DNA(D.I.)	L.I.	DNA(D.I.)	L.I.	DNA(D.I.)
1	NE	A(1.84)	NE	A(1.80)	NE	A(1.82)						
2	8.1	NE	20.4	NE	32.5	NE						
3	NE	NE	NE	NE	NE	NE						
4	10.1	D	12.8	D	13.5	D						
5	12.5	D	25.2	A(2.03)	29.6	A(1.84)						
6	8.8	D	40.4	A(1.58)	38.0	A(1.66)	34.4	A(1.53)				
7	11.2	A(1.68)	15.5	A(1.82)	15.3	A(1.85)	17.6	D	9.2	NE		
8	9.5	D	8.8	A(1.87)	8.9	A(2.05)						
9	20.3	D	23.4	D	31.5	A(1.65)	45.5	D			48.2	NE
10	7.5	NE	35.6	NE	42.3	NE	38.5	D				
11	8.5	D	25.6	A(1.84)	33.7	NE	23.5	A(1.87)	9.8	NE		
12	15.3	ND	28.4	ND	19.3	ND	48.4	ND				
13	8.3	ND	15.8	ND	12.1	ND						
14	18.8	ND	20.3	ND	25.3	ND						
15	15.7	ND	45.3	ND	47.2	ND	44.4	ND				

L.I.: labeling index of Ki-67
DNA((D.I.): DNA ploidy pattern (DNA index)
D: diploidy pattern
A: aneuploidy pattern

P: peripheral component
I: intermediate component
C: central component
NE: not evaluable
ND: not done

shown in Table 2. In each of the components of the primary tumor and metastatic lesions, immunoreactivity to DO-7 higher than grade 2 correlated well with mutation of the p53 gene identified by PCR-DGGE analysis, except for 4 lesions examined. On the other hand, mutation was not detected for lesions of grade 0 and 1. From these results, it was considered that immunoreactivity to DO-7 of grade 2 and above should actually be evaluated as the mutant oncoprotein, and that immunoreactivity of grade 1 might indicate an oncoprotein of the wild type.

Immunohistochemical findings on Ki-67 antigen (Table 3):

The immunohistochemical findings on Ki-67 antigen in case no. 6 is shown in Fig. 5, and a summary of Ki-67 analysis is shown in Table 3. In most cases the central and intermediate components had a greater index than the peripheral one. In addition, the grade for the metastatic tumor in the lymph nodes and brain was similar to that of the central and intermediate components, but was greater than that of the peripheral component. This is best exemplified in cases no. 6, 7, 9, 10, 11 and 15. However, the indices of metastatic lung tumors, shown in cases no. 8 and 11, resembled that of the peripheral component, and were lower than those of the central and intermediate components.

Flow cytometrical analysis on DNA (Table 3):

Flow cytometrical analysis on the DNA content of cells was performed in 11 cases and valuable results were obtained from 8. The result from case no. 6 is shown in Fig. 6. In the peripheral component, the adenocarcinoma cells demonstrated a diploidy pattern, while those in the intermediate and central components as well as in the metastasis of the lymph node showed an aneuploidy pattern. Other cases also showed a variety of patterns in each of the components of the primary tumor and metastatic lesions. For example, in cases no. 5 and 8, both the central and intermediate components showed an aneuploidy pattern with similar DNA indices, while the peripheral component showed a diploidy pattern. In cases no. 6 and 11, the metastasis of the lymph nodes showed an aneuploidy pattern which resembled those of the central and intermediate components.

Correlation among p53 analysis, Ki-67 immunohistochemistry and DNA ploidy pattern:

As mentioned above, mutation of the p53 gene was detected more frequently in the intermediate and central components of the primary tumors; moreover, in these components, the L.I. for Ki-67 was greater, and the DNA showed an aneuploidy pattern. In case no. 6, greater indices for Ki-67 and DNA anuploidy pattern were shown in both the central and intermediate components, and mutation of the p53 gene was detected in the central component. In the other cases, the L.I. for Ki-67 and DNA ploidy pattern correlated relatively well with the mutation of the p53 gene in each component.

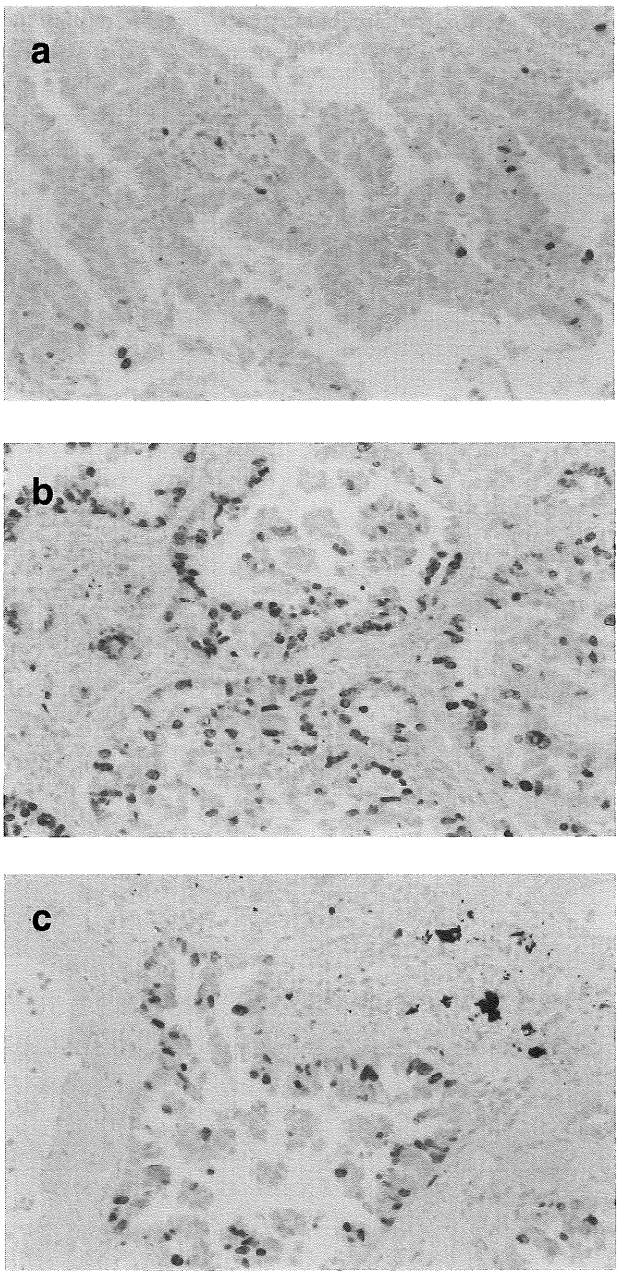

Fig. 5. (a) Peripheral component of case no.6. A few carcinoma cells showed immunoreactivity of Ki-67 and its labeling index was 8.8 (SAB method, ×200).
(b) Intermediate component of case no.6. Many carcinoma cells showed immunoreactivity of Ki-67 and its labeling index was 40.4 (SAB method, ×200).
(c) Central component of case no.6, Many carcinoma cells showed immunoreactivity of Ki-67 and its labeling index was 38.0 (SAB method, ×200).

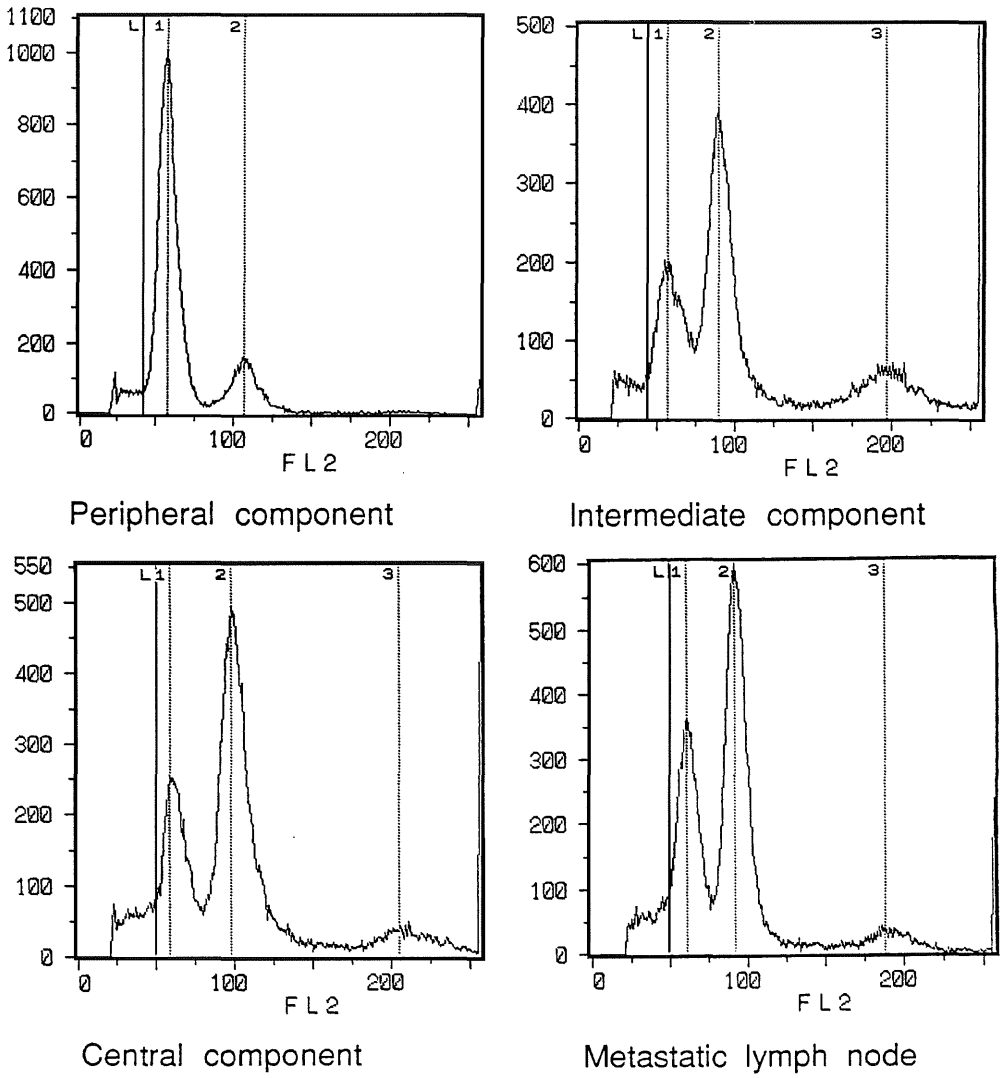

Fig. 6. DNA ploidy pattern in each component of case no.6: In the peripheral component tumor cells demonstrate a diploidy pattern, while those in the intermediate and central component as well as in the metastasis of the lymph node show an aneuploidy pattern.

DISCUSSION

The finding that the level of Ki-67, marker of the growth fraction, was high in the central and intermediate components indicate that many carcinoma cells in these components stay in the growth-cycle (G_1 to M phase). The frequent occurrence of an aneuploidy pattern of DNA in these components indicates that the carcinoma cells have high malignant potential. Another noticeable finding is that the metastatic carcinoma cells in the lymph nodes show an aneuploidy pattern, having a DNA index similar to that in the central or the intermediate component, suggesting that metastatic cells spread from these components. Thus, it is deduced that carcinoma cells in the central and intermediate components retain more malignant biological potential and a higher metastatic potential than those in the peripheral component.

A high incidence of mutation of the p53 gene was found in the central and intermediate components by means of the immunohistochemical and PCR-DGGE methods. Taking into account the above mentioned results, I conclude that mutation of the p53 is intimately related to the malignant potential of adenocarcinoma of the lung. This is in accord with the notion of Nowell that the process of tumor progression generates from an acquired genetic lability and that the oncogenes or tumor suppressor genes play a specific role in this process[11]. Recent studies have shown that the wild type of p53 gene regulates transcription through sequence-specific DNA binding, and mutation of the p53 gene abrogates this activity, leading to uncontrolled proliferation of the affected cells[8,14,22]. In other words, the p53 gene controls the transition of resting normal cells from the G_0-state to the G_1-state and the prolifer-

ation of actively growing cells in the late G_1-state after mitogenic stimulation[15]. There are several reports on the close correlation of mutation of the p53 gene in cancer and patients' prognosis. Hori et al examined 71 non-small cell lung cancers by the PCR-SSCP method, and concluded that mutation of the p53 gene is related to the patients' poor prognosis[4]. Quinlan et al also showed that in 114 non-small cell lung cancers accumulation of p53-oncoprotein in the nuclei correlates with poor prognosis by means of the immunohistochemical method[13]. They suggested in the same paper that p53-oncoprotein accumulates at the early step of carcinoma. This was based on results that in the metastatic carcinomas of lymph nodes an accumulation of p53-oncoprotein was observed in 7 out of 24 stage II cases, although the primary lesions were negative for p53-oncoprotein. By an immunohistochemical study, Hiyoshi et al observed that p53-oncoprotein densely expresses in patchy spots within one tumor, and presented the hypothesis that mutation of the p53 gene takes part in the progression of lung adenocarcinoma[3]. In a previous study, we also demonstrated that mutation of the p53 gene is an independent prognostic factor in small-sized adenocarcinomas of the lung by the immunohistochemical and PCR-DGGE methods[21].

Making allowance for the general belief that lung adenocarcinoma cells differentiate from the peripheral to the central region and mutation of the p53 gene loses the ability to control cell growth, leading to the generation of lung carcinoma, I speculate that mutation of the p53 gene may be related with the progression of tumor cells in adenocarcinoma of the lung.

Nomori et al took an approach similar to ours and divided the primary lesions in 20 cases of differentiated lung adenocarcinoma into three components, type 1, type 2, and type 3, corresponding to the peripheral, intermediate and central components in my classification. By cytofluorometric DNA analyses, the type 2 areas were shown to have a greater nuclear DNA content (NDC) than the type 1 areas and there was a similarity of mean NDC between type 2 and distant metastatic lesions. They also proposed that malignant cells are confined to the type 2 areas in the primary lesion[9].

Another noticeable point in the present study is that carcinoma cells in localized metastasis in the lung have a lower proliferative activity and a diploidy pattern without mutation of the p53 gene. These characteristics are also recognized in carcinoma cells in the peripheral components of the primary tumor. As the "seed and soil" theory suggests[12], carcinoma cells with a lower malignant potential in the peripheral component may be able to metastasize within the lung. The route of metastasis is probably via the lymphatics, because metastatic carcinoma was very often localized in the subpleural region, and carcinoma emboli in the lymphatics were seen surrounding the metastatic tumor. Nomori et al also showed in seven cases of well-differentiated lung adenocarcinomas with localized lung metastases that the carcinoma cells examined by cytofluorometric analyses had significantly lower DNA content than those of 21 cases of lung adenocarcinoma with both lung and distant metastases. From these results, they concluded that well-differentiated adenocarcinoma with selective lung metastasis had a lower malignant potential[10].

In the near future, a more precise examination is necessary to characterize the carcinoma cells in the intermediate and central areas of adenocarcinoma of the lung which show more malignant features, in order to clarify multistep carcinogenesis in adenocarcinoma of the lung.

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