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Accumulation of Losses of Heterozygosity and Multistep Carcinogenesis in Pulmonary Adenocarcinoma[1]

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ABSTRACT

Sixty-six replacing growth-type early lung adenocarcinomas, measuring 2 cm or less across their greatest dimension, were used to investigate allelic losses at eight loci on the eight chromosomes carrying the principal cancer-associated genes. In total, 2 (16.7%) of 12 type A tumors (localized bronchioloalveolar carcinoma, LBAC) and 11 (39.3%) of 28 type B tumors (LBAC with alveolar collapse), which correspond to early lung adenocarcinomas including cancers *in situ*, showed allelic losses in one or more of the regions examined. In contrast, 25 (96.2%) of 26 type C tumors (LBAC with active fibroblastic proliferation), which correspond to small but advanced tumors, showed allelic losses in one or more regions. The change in histology from type A to type C was characterized by a significant rise in the incidence of allelic losses ($P < 0.01$). Deletions of 3p, 17p, 18q, and 22q increased significantly during malignant progression. In type C tumors that showed heterogeneous histological features, the tumor cells in the central fibrotic areas exhibited more allelic losses than those in the peripheral bronchioloalveolar growths and were, therefore, considered to have progressed to a more advanced stage than the tumor cells in the peripheral regions.

INTRODUCTION

Lung cancer is the leading cause of cancer death among men and women in the United States and has recently become one of the most common malignancies in Japan. It can be subdivided into four major histological subtypes: squamous cell carcinoma, adenocarcinoma, large cell carcinoma, and small cell carcinoma (1). Small cell carcinoma has unique clinical and histological characteristics and is classified separately. Of the NSCLCs,[3] the two major histological subtypes are squamous cell carcinoma and adenocarcinoma. There is now a consensus that squamous cell carcinoma is strongly related to cigarette smoking, and the sequence of histological changes from dysplasia and *in situ* carcinoma to invasive carcinoma has been well established for this subtype (2). Recently, Wistuba *et al.* reported that sequential molecular abnormalities are involved in the multistep development of squamous cell carcinoma of the lung (3).

Unlike squamous cell carcinoma, the other major histological subtype of NSCLC, lung adenocarcinoma, is characterized by histological and cytological heterogeneity: indeed, a mixture of several different cell types is present in advanced tumors. Therefore, it has been very difficult to characterize the sequential progression of lung adenocarcinoma on a morphological basis. Recently, Noguchi *et al.* subdivided small, early adenocarcinomas of the lung (*i.e.*, ≤2 cm in diameter) into two groups using histological criteria (4). In one of these subtypes, the tumor growth replaces the pulmonary alveolar structure (replacing growth type), whereas in the other, the tumor growth is nonreplacing and destructive. Replacing growth-type adenocarcinoma, which is the major histological subtype including 75% of all small adenocarcinomas, can be further subdivided into three subgroups (Fig. 1). Type A, or LBAC, is sometimes difficult to distinguish from the precancerous lesion, atypical adenomatous hyperplasia. Type B comprises LBACs with foci of alveolar structural collapse. These two subgroups show no lymph node metastasis and have a 5-year survival rate of 100%. On the other hand, 28% of type C tumors (that is, LBACs with active fibroblastic proliferation) do show lymph node metastasis, and their 5-year survival rate is 74.8%. These clinicopathological and biological findings suggest a sequential progression from type A and B to type C tumors during the course of pulmonary adenocarcinogenesis.

Several studies have used mutation analysis or RFLP analysis to study recessive oncogenes, such as *p53*, *p16*, and *Rb*, and to investigate the abnormal expression of oncogenes, such as *ras*, *myc*, and *erb*B 2 (5, 6). However, relatively little is known about the sequence of molecular events that precedes the development of invasive lung adenocarcinoma. In this study, we used small, early lung adenocarcinomas showing replacement growth to investigate LOH on eight chromosomes, and we studied the sequential development of the molecular abnormalities involved in the multistep pathogenesis of lung adenocarcinoma.

MATERIALS AND METHODS

Specimens and Histological Typing. Sixty-six replacing growth-type lung adenocarcinomas measuring ≤2 cm across their greatest dimension were obtained from patients undergoing surgical resection between 1997 and 1999 at the National Cancer Center Hospital East, Chiba, Japan. The specimens were fixed with methanol and embedded in paraffin. On the basis of our previously proposed criteria, these small lung adenocarcinomas were subdivided into three histological groups: type A, LBAC (12 specimens); type B, LBAC with foci of alveolar structural collapse (28 specimens); and type C, LBAC with foci of active fibroblastic proliferation (26 specimens; Fig. 1; Ref. 4). Clinically, LBAC and LBAC with foci of alveolar structural collapse (that is, types A and B) are considered to be *in situ* peripheral adenocarcinomas with a good prognosis, whereas LBAC with foci of active fibroblastic proliferation (type C) appears to be a relatively early stage of adenocarcinoma but is more advanced than types A and B.

Microdissection Analysis. For the microdissection analysis, two or three 10-μm-thick sections from each specimen were deparaffinized and stained with hematoxylin. The stained sections were dried; then the tumor cells and normal cells, such as lymphocytes or bronchial epithelial cells, were microdissected separately, using a Pixcell Laser Capture Microdissection system (Arcturus Engineering Inc., Mountainview, CA; Ref. 7). Finally, 100–200 tumor cells and the same number of normal cells were microdissected from each specimen, and their genomic DNA was extracted. For type B and C tumors, tumor cells from the LBAC lesions and those from the collapsed or fibrotic areas were microdissected separately.

Multiplex PCR-LOH analysis. To evaluate LOH, we used 19 dinucleotide microsatellite repeat polymorphisms located at the following sites in the eight genes reported to play a major role in human carcinogenesis (8–16): *FHIT* (3p;

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[3] The abbreviations used are: NSCLC, non-small cell lung carcinoma; LBAC, localized bronchioloalveolar carcinoma; FRL, fractional regional loss.

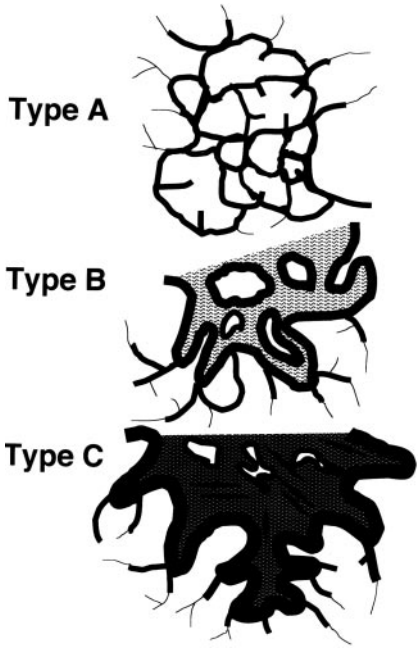

Fig. 1. Scheme of replacing growth type adenocarcinoma of the lung. *Type A*, LBAC. *Type B*, LBAC with focus of collapse of alveolar structure. *Type C*, LBAC with focus of active fibroblastic proliferation. (See Ref. 4.)

D3S1300, D3S1312, and D3S1313), *APC* (5q; D5S346 and D5S82), *p16* (9p; D9S171 and D9S162), *Int-2* (11q; INT-2), *Rb* (13q; D13S270, D13S273 and D13S176), *p53* (17p; TP53 and D17S520), *Smad* 4 (18q; D18S46, D18S363 and D18S474), and Band M (22q; D22S1140, D22S1170 and D22S1161).

Data Analysis. For the analysis of LOH, we used two approaches: (*a*) for the individual foci examined, we determined the frequency of LOH within the relevant chromosomal region; and (*b*) to correlate morphological changes with allelic losses, we calculated the mean FRL index as follows (3):

$$\text{FRL} = \frac{\text{Total number of chromosomal regions with LOH}}{\text{Total number of informative regions}}$$

For both approaches, Fisher's exact test and Student's *t* test were used for the statistical analysis.

RESULTS

Sixty-six small, replacing growth-type lung adenocarcinomas were classified by their histological features into types A, B, and C (Fig. 1 and Fig. 2*a*, *c*, *d*, *f*, and *g*). Clinicopathologically, none of the type A or B tumors (40 tumors) showed lymph node metastasis, so they were classified as $pT_1N_0M_0$ according to the TNM classification (Table 1). On the other hand, 7 (26.9%) of the 26 type C tumors showed pleural invasion, mediastinal lymph node metastasis, or malignant pleural effusion; therefore, they were classified as $pT_2N_0M_0$, $pT_3N_0M_0$, $pT_1N_1M_0$, $pT_1N_2M_0$, $pT_4N_0M_0$ or $pT_4N_2M_0$. These clinicopathological findings are summarized in Table 1. There are significant differences in the mean diameter of the tumor and frequencies of lymphatic, vascular, and pleural invasions and mitotic index between type A and B tumors and type C tumors. Most of the patients are still alive and have had no recurrence of their carcinoma; however, two of those with type C tumors died of tumor progression.

We detected allelic losses in one or more chromosomal regions in 2 (16.7%) of the 12 type A tumors, 11 (39.3%) of the 28 type B tumors, and 25 (96.2%) of the 26 type C tumors (Table 2; Fig. 2*b*, *e*, and *h*). The overall frequency of allelic losses was significantly lower in type A and B tumors than in type C tumors ($P < 0.001$). The FRL indices for type A and B tumors (0.03 and 0.10) were also significantly lower than that for type C tumors (0.43); ($P < 0.001$; Fig. 3). Furthermore, the change in histology from type A to type C was characterized by a significant rise in the FRL indices.

Allelic losses did not occur in a random fashion. The frequencies of allelic losses at chromosomal loci 3p, 17p, 18q. and 22q were significantly higher in type C tumors than in type A and B tumors (Table 2). On the other hand, there were no significant differences in the frequencies in allelic losses at the 5q, 9p, 11q, and 13q loci between type C tumors and type A and B tumors. Thus, the progressive histological changes from type A through type B to type C was accompanied by a significant increase in the frequency of allelic losses at 3p, 17p, 18q, and 22q. In particular, allelic losses at 11p and 22q were recognized only in type C tumors.

The central areas of the tumors, which showed fibrotic changes or collapse of alveolar structure, and the peripheral areas, which showed bronchioloalveolar spreading, were examined separately for type B and C tumors (Figs. 1 and 2*c–h*). Seven (25.0%) of 28 type B tumors and 12 (46.2%) of 26 type C tumors showed different allelic loss patterns in one or more of the regions examined between the tumor cells in the central region and those in the peripheral regions (Table 3). Among 10 regions of the seven type B tumors, four (40.0%) showed allelic losses only in the tumor cells within the foci of collapse. On the other hand, among 15 regions of the 12 type C tumors, 12 (80.0%) showed allelic losses only in the tumor cells within the central fibrotic area. Thus, in type C tumors, allelic loss events occurred more frequently in the tumor cells within the central fibrotic area than in the tumor cells within the peripheral region.

Most type A tumors (83%) showed no allelic losses in any of the regions examined, although two of those tumors showed allelic losses in a single region (9p and 17p, respectively). However, we could not find any histological and cytological differences between the type A tumors with and without allelic losses. On the other hand, 25 of the 26 type C tumors showed allelic losses in one or more chromosomal regions. Only one type C tumor showed no allelic loss in any region. Histologically, the latter tumor showed no hyalinization but exhibited solid growth of the tumor cells within the central area of fibrosis.

DISCUSSION

In many organs, carcinogenesis has been interpreted as a multistep process because of the accumulation of several sequential molecular abnormalities. Wistuba *et al.* (3) analyzed the pathogenesis of pulmonary squamous cell carcinoma arising in respiratory epithelium damaged by smoking and reported a progressive increase in the overall frequency of LOH within the cell clones as the severity of the histopathological changes increased from hyperplasia through dysplasia to carcinoma *in situ*. In the present study, we focused on another major histological subtype of lung cancer, adenocarcinoma. We previously reported the concept that peripheral adenocarcinoma of the lung undergoes sequential progression from atypical adenomatous hyperplasia through LBAC to small but advanced LBAC with fibroblastic proliferation (Figs. 1 and 2; Refs. 4, 17). Our current findings confirm and extend these previous morphological observations, showing that the incidence of LOH increases as lung adenocarcinoma undergoes histological progression.

It was of particular interest that, among the eight chromosomal regions examined, the incidences of allelic losses at 3p, 17p, 18q and 22q were significantly lower in type A and B tumors than in type C tumors. Using stage I primary and metastatic NSCLCs, Shiseki *et al.* examined LOH at 84 loci on 22 autosomal chromosomes by RFLP (18). They demonstrated that allelic losses at 3p, 13q, and 17p were involved in the genesis of NSCLC, whereas losses at 2q, 9p, 18q, and 22q played an important role in its

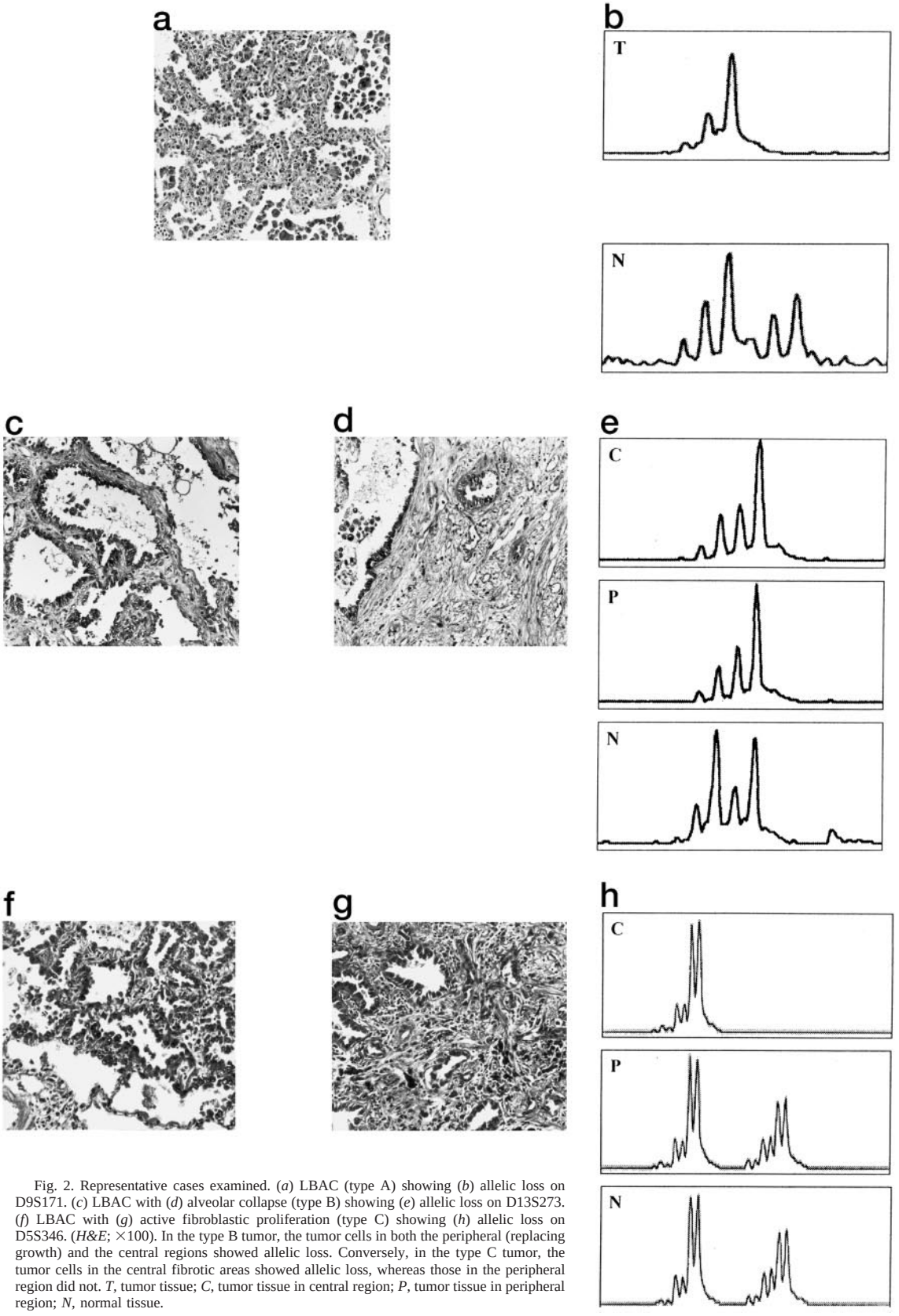

Fig. 2. Representative cases examined. (*a*) LBAC (type A) showing (*b*) allelic loss on D9S171. (*c*) LBAC with (*d*) alveolar collapse (type B) showing (*e*) allelic loss on D13S273. (*f*) LBAC with (*g*) active fibroblastic proliferation (type C) showing (*h*) allelic loss on D5S346. (*H&E*; ×100). In the type B tumor, the tumor cells in both the peripheral (replacing growth) and the central regions showed allelic loss. Conversely, in the type C tumor, the tumor cells in the central fibrotic areas showed allelic loss, whereas those in the peripheral region did not. *T*, tumor tissue; *C*, tumor tissue in central region; *P*, tumor tissue in peripheral region; *N*, normal tissue.

progression. Wistuba *et al.* also detected frequent allelic loss at 3p during the genesis of squamous cell carcinoma (3). In contrast, our present results indicate that allelic loss at 9p is an early event in the genesis of adenocarcinoma, whereas loss at 3p and 17p are relatively late events. These discrepancies between our results and those of previous studies might be attributable to the differences of regions examined at one chromosome and/or the histological heterogeneity of NSCLC: squamous cell carcinoma is a distinct his-

tological subtype of lung carcinoma, whereas the umbrella term NSCLC includes not only adenocarcinoma but also squamous cell carcinoma and large cell carcinoma. Recently, Wistuba *et al.* (19) examined allelic losses at many regions of chromosome 3p in lung cancer of all types and in bronchial precancerous lesions. They found frequent losses of 3p at one or more of multiple sites in these lesions, and they also showed 3p14 (FHIT region) is lost relatively late among the regions examined. Taken together, these present and previous findings of Wistuba *et al.* suggest that allelic losses at various sites of chromosome 3p except 3p14 are frequent events in early lesions of lung carcinoma. On the other hand, according to our present results, allelic losses at 5q, 9p, 11q, and 13q occur at a similar frequency in all early stages of adenocarcinoma. Therefore, abnormalities of the tumor suppressor genes *APC*, *p16*, *Int-2*, and *Rb* might be associated with the early stages of adenocarcinogenesis.

Table 1 *Comparison of the clinicopathological features of the small adenocarcinomas examined*

	Types A and B	Type C	*P*
No. of specimens	40	26	
Mean diameter, across maximum cut surface of the tumor (cm)	1.20	1.77	<0.0001
p[a]			
$T_1N_0M_0$	40	19	<0.0001
$T_1N_0M_0$	0	7	
ly factor			
−	39	17	0.0006
+	1	9	
v factor			
−	40	17	<0.0001
+	0	9	
Pleural invasion			
−	40	17	<0.0001
+	0	9	
MI			
1	38	19	0.0308
2	2	5	
3	0	2	

[a] p, pathological stage; ly factor, invasion of lymphatic duct; v factor, invasion of blood vessel.
[b] MI, mitotic index. 1, less than 1 per 10 HPF (high power fields); 2, 1–5 per 10 HPF; 3, >5 per 10 HPF.

Table 2 *Allelic losses in small adenocarcinoma of the lung*

	Type, *n*			
Locus (Gene)	A[a]	B[b]	C[c]	*P* (A & B:C)
3p	0/10	1/27	13/25	<0.001
(*FHIT*)	(0%)	(3.7%)	(52.0%)	
5q	0/8	3/23	5/20	0.237
(*APC*)	(0%)	(13.0%)	(25.0%)	
9p	1/7	2/15	5/12	0.098
(*p16*)	(14.3%)	(13.3%)	(41.7%)	
11q	0/7	0/14	2/9	0.083
(*Int-2*)	(0%)	(0%)	(22.2%)	
13q	0/9	5/24	9/23	0.061
(*Rb*)	(0%)	(20.8%)	(39.1%)	
17p	1/8	3/24	10/20	0.005
(*p53*)	(12.5%)	(12.5%)	(50.0%)	
18q	0/11	3/25	15/26	<0.001
(*Smad* 4)	(0%)	(12.0%)	(57.7%)	
22q	0/9	0/25	7/17	<0.001
(*Band M*)	(0%)	(0%)	(41.2%)	
Any region	2/12	11/28	25/26	<0.001
	(16.7%)	(39.3%)	(96.2%)	
FRL index	2/69	17/177	66/152	<0.001
	(0.03)	(0.10)	(0.43)	

[a] Type A, LBAC.
[b] Type B, LBAC with alveolar collapse.
[c] Type C, LBAC with active fibroblastic proliferation.

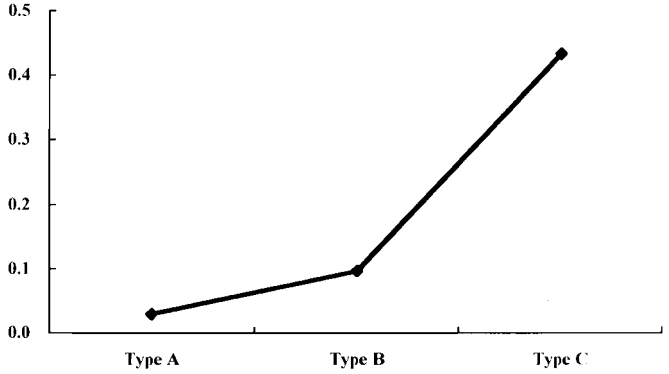

Fig. 3. FRL indices in eight chromosomal regions for type A, B, and C tumors.

Table 3 *Heterogeneous pattern of allelic losses in Type B and Type C tumors*

Type	No. of specimens	No. of specimens showing different allelic loss patterns	No. of loci showing loss only in the central area ÷ no. of loci showing different allelic loss patterns
B	28	7 (25%)	4/10 (40.0%)
C	26	12 (46.2%)	12/15 (80.0%)

In type B and type C tumors, we found different allelic loss patterns between the tumor cells in the areas of alveolar collapse or fibrosis and those in the peripheral region, which showed replacing growth. These findings indicated that at least two clones are already present in early adenocarcinomas that are <2 cm in diameter. On the other hand, it was interesting that the incidence (40.0%) of allelic losses in tumor cells within the areas of collapse in type B tumors was much lower than that (80.0%) within the fibrotic areas of type C tumors (Table 3). Thus, the significantly greater frequency of allelic losses within the fibrotic areas than in the peripheral regions of type C tumors indicates that the tumor cells in the fibrotic areas have progressed to a more advanced stage than those in the peripheral regions.

On the basis of our findings, pulmonary adenocarcinogenesis can be characterized as follows: (*a*) deletions at chromosomal loci 5q, 9p, 11q, and 13q are relatively early events, which suggests that inactivation of the *APC*, *p16*, *Int-2*, and *Rb* genes might be functionally associated with pulmonary adenocarcinogenesis; (*b*) deletions of 3p, 17p, 18q, and 22q increase significantly during the course of malignant progression; and (*c*) in type C tumors with heterogenous histological features, the tumor cells in the fibrotic areas are more advanced and malignant than those in the peripheral regions, which show replacing growth of the bronchioloalveolar structure. These findings of the accumulation of multiple allelic losses confirm our previous morphological observations of multistage carcinogenesis in pulmonary adenocarcinoma.

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