Proliferative Activity, p53 Expression and Loss of Heterozygosity on 3p, 9p and 17p in Atypical Adenomatous Hyperplasia of the Lung

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ABSTRACT

We examined the preneoplastic nature of atypical adenomatous hyperplasia (AAH) of the lung by comparing the proliferative activity, p53 oncosuppressor gene product and loss of heterozygosity (LOH) on 3p, 9p and 17p of 20 AAH lesions (8 cases) with corresponding normal peripheral lung tissue and adenocarcinoma from the same cases. Analysis of proliferative activity with the Ki-67 labelling index and argyrophilic nucleolar organizer regions (AgNORs) score indicated that AAH had a proliferative activity intermediate between that of normal cell and adenocarcinoma. Although low level expression of p53 was detected in 7 AAH lesions, the intensity of p53 expression in AAH was weaker than that in carcinomas. Microsatellite analysis of chromosome 3p, 9p and 17p showed LOH of 18%, 13% and 6% respectively in the AAH lesions, while the corresponding carcinomatous lesions showed LOH of 67%, 50% and 17% respectively. All AAH lesions that showed LOH had moderate or severe histological atypia. One AAH lesion with moderate atypia showed LOH both on 3p and 17p. In conclusion, these results indicated that AAH lesions with moderate or severe atypia may show the preneoplastic stage of lung adenocarcinoma.

Key words: Atypical adenomatous hyperplasia, Adenocarcinoma, Lung, Microsatellite instability

Recently, lung adenocarcinomas have been increasing in Japan as well as in Western countries[31,36]. However, their etiology is not well understood. To determine the histogenesis and etiology of lung adenocarcinoma, it is helpful to analyze the biological nature of its preneoplastic lesion.

The peculiar lesion, named atypical adenomatous hyperplasia (AAH)[27] or bronchioloalveolar cell adenoma[17] of the lung, has been reported as a preneoplastic lesion. AAH lesions are frequently observed close to the primary adenocarcinoma, or as independent lesions in a lung with adenocarcinoma, whereas they are rare in lungs with squamous cell carcinoma or undifferentiated carcinoma. In fact, it was reported that the frequency of AAH detected is 18.8% in lung adenocarcinoma cases, which is higher than in other histological types of carcinoma[15]. In another report, the frequency of AAH was 8.8% in 285 cases of lung adenocarcinoma[11]. Immunohistochemical and ultrastructural studies have demonstrated that AAH consists of Clara cells and type II alveolar cells[19,20]. Several morphometric analyses have demonstrated that the

mean nuclear area (MNA) and the standard deviation (SD) of the MNA are lower in AAH than in adenocarcinoma[13,14], and this method is useful for the diagnosis of AAH. In spite of these reports, the biological characteristics of AAH so far remain unclear.

The purpose of the present study, therefore, was to determine its biological nature with special reference to its preneoplastic features by analysing its proliferative activity as well as its genetic alterations.

MATERIALS AND METHODS
Detection of AAH

AAH lesions were obtained from 8 surgically resected non-small cell lung cancers (6 adenocarcinomas, 1 adenosquamous carcinoma and 1 double cancer case with adenocarcinoma and squamous cell carcinoma). The clinicopathological data of the 8 cases are shown in Table 1. Four out of eight patients were smokers (smoking index: 150–1600). The lung tissues were fixed with 10% buffered formaline, and serial step cut sections of

Table 1. Clinicopathological Aspects of Patients with AAH Lesions

Patient No.	AAH No.	Age	Sex	Histology of Primary Carcinoma	Smoking index
1	1	56	F	Well diff. adca*, bronchiolo-alveolar type	0
2	2	59	M	Well diff. adca, tubular type	0
3	3	59	M	Adenosquamous carcinoma	150
4	4	80	F	Well diff. adca, papillary type	0
5	5	62	M	Mod. diff. adca †, papillary type	450
	15				
6	16	67	M	Mod. diff. adca, tubular type	1125
	17				
7	18	77	M	Mod. diff. sq ca ‡ and well diff. adca, papillary type	0
8	19	61	M	Well diff. adca, papillary type	1600
	20				

* Well differentiated adenocarcinoma
† Moderately differentiated adenocarcinoma
‡ Moderately differentiated squamous cell carcinoma

resected lung tissue were made. Each AAH lesion was observed by four independent pathologists and the diagnosis of suspicious lesions was discussed under the same microscope. The criteria used to diagnose AAH were those of Miller[17], Nakanishi[20] and Mori[18]. Briefly, every lesion was small and microscopically isolated from the primary lung tumors. The lesions consisted of non-ciliated cuboidal cells single-lying on the alveolar septa. Histologically, the cells resembled Clara cells and/or type II alveolar cells. In every lesion, the alveolar structure was well preserved. The degree of atypia was scored as "mild", "moderate" or "severe" according to the cellular and structural atypia (Fig. 1). The main check points of cellular atypia were as follows: the size of nuclei, irregularity of nuclear size and loss of polarity. Using these criteria, 20 AAH lesions were detected. Five patients (patients 1, 2, 3, 4 and 7) had one AAH lesion, and three patients had multiple lesions; none of the lesions was detected before surgery. The size of the lesions was measured using a micrometer.

Ki-67 labelling index
To evaluate proliferative activity, the AAH lesions were stained with anti-Ki67 monoclonal antibody, MIB-1 (Immunotech, Marseilles, France), at a dilution of 1: 50. Histological sections were prepared from formaline-fixed, paraffin-embedded tissue blocks, and then deparaffinized. After blocking endogenous peroxidase, the sections were placed in 10 mM citric acid and treated with microwaves. The immunoreactive compounds were detected using a Histofine ABC-Kit (Nichirei, Tokyo, Japan) and were visualized with diaminobenzidine (Dojin Laboratories, Kumamoto, Japan). The nuclei were counterstained with hematoxylin. The labelling index (%) of Ki-67 positive cells was calculated by

counting 1000 cells, and the result was compared with those of normal peripheral alveolar cells and carcinoma cells. The correlations between the Ki-67 labelling index, the size of the lesions and the degree of atypia were analyzed statistically.

Argyrophilic nucleolar organizer regions (AgNORs)
AgNORs staining was conducted using Ploton's one step method[25]. The average number of AgNORs per nucleus was calculated by counting 1000 nuclei for each lesion.

Expression of p53
The monoclonal antibody DO-7 (Novocastra, Newcastle, UK), which reacts with both the wild-type and mutant forms of p53, was used for immunohistochemistry at a dilution of 1: 30. The immunostaining method was almost the same as that mentioned above for the anti-ki67 monoclonal antibody. The staining pattern was scored according to the percentage of tumor cells stained: +++, marked, more than 20% of the tumor cells were stained; ++, moderate, 10%–20% stained; +, mild, less than 10% stained; - , no staining.

Genetic analysis
1. Microdissection and DNA Extraction
Sections $10 \mu m$ thick were cut from the paraffin blocks and deparaffinized in xylene. The lesions (AAH lesions, cancerous lesions and normal peripheral lung lesions) were scraped using a 27 gauge needle under a dissecting microscope taking care to avoid contamination by the surrounding tissue, and placed in 100% ethanol. After centrifuging, the samples were dried in a vacuum. The scraped tissue was digested with proteinase K and extracted with phenol/chloroform before the DNA was precipitated with ethanol.

2. DNA Probes and PCR-Based Loss of Heterozygosity (LOH) Analysis

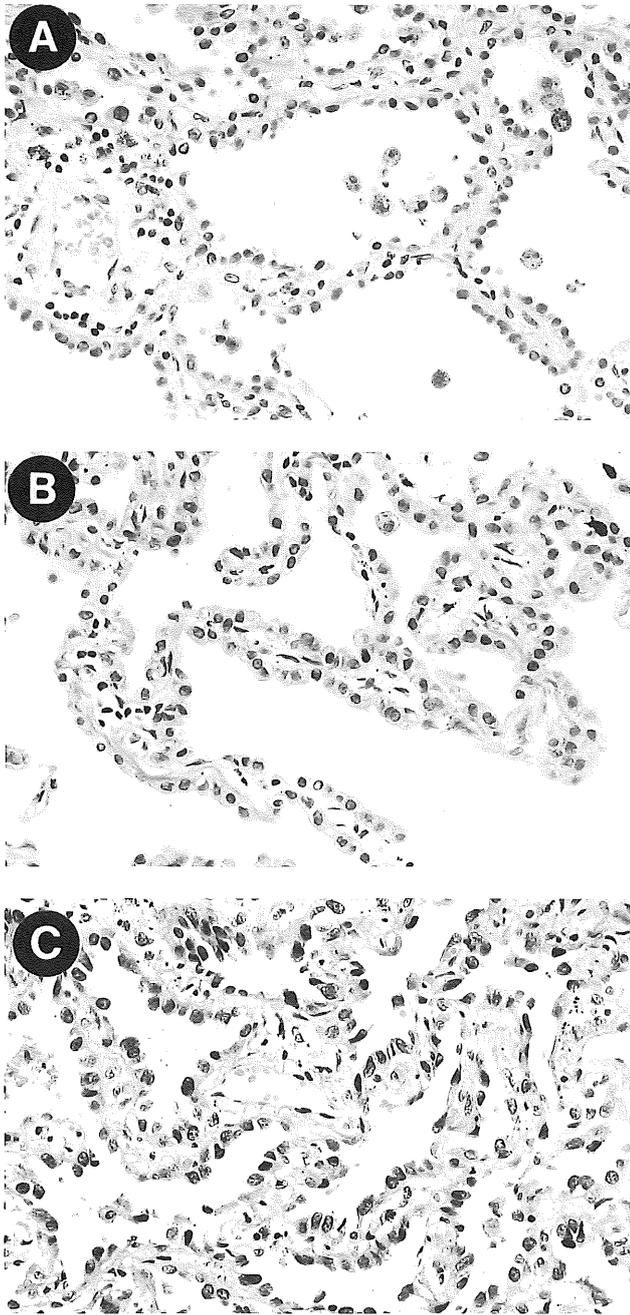Using microsatellite markers, we detected LOH on 3p, 9p and 17p. The microsatellite markers

Fig. 1. Histological features of AAH lesions (Hematoxylin-eosin stain; original magnification ×100.)

(A) AAH with mild atypia (Lesion 12). The cells with small nuclei are lying regularly on the alveolar septa.

(B) AAH with moderate atypia (Lesion 10). The lining cells have irregular sized-nuclei.

(C) AAH with severe atypia (Lesion 2). The lining cells have enlarged and hyperchromatic nuclei compared with those in AAH lesions with mild or moderate atypia.

used were *D3S643* and *D3S663* on chromosome 3p21.3, *D3S1228* on chromosome 3p14.1–14.3[6,9,22], interferon alfa (*IFNA*) and *D9S171* on chromosome 9p21–22, *D9S144* on chromosome 9p22[2,16,33], and *TP53* on chromosome 17p13[10].

Polymerase chain reaction (PCR) was performed in a $10\mu l$ reaction volume containing 10 pmol of each primer, $1.0\mu l$ 10 × buffer (1 × 10 mM Tris (pH 8.8), 50 mM KCl, 1.5 mM MgCl$_2$, 0.1% Triton X-100), dNTP (2.5 mM dATP, 2.5 mM dTTP, 2.5 mM dGTP, 2.5 mM dCTP), $0.05\mu l$ Taq polymerase (5 U/μl), 5μCi [α -^{32}P] dCTP and $0.5\mu l$ of template DNA. PCR was performed at an initial temperature of 94C° for 5 min, followed by 35 cycles consisting of 1 min at 94C°, 2 min at 60C° (annealing temperature) and 1 min at 72C°, with the final extension for 10 min at 72C°. The PCR products were electrophoresed on a 6% polyacrylamide gel for 1–2 hours at 60 W. Autoradiography was conducted for 1–3 days.

We also conducted a PCR-RFLP (restriction fragment length polymorphism) assay to detect the *D3S2* locus located on 3p21 using essentially the same protocol as described previously[4].

Statistical analysis

The Student's *t*-test and Fisher's exact test were used to analyze differences between the two respective groups. A P-value of less than 0.05 was considered significant. The Kruskal-Wallis test (nonparametric one-way analysis of variance) was used to examine the between-group variation. The Spearman's correlation coefficient was used to elucidate the correlation of the two groups.

RESULTS

Size of AAH according to the atypia (Table 2)

The size of the AAH lesions ranged from 1 to 12 mm (average: 3.4 mm). The average size of AAH lesions increased as the degree of atypia increased (mild vs severe, p<0.05 and moderate vs severe, p<0.01, Student's *t*-test). The Kruskal-Wallis test also indicated significant differences according to the degree of atypia.

Table 2. Average Size of AAH Lesions

	AAH			
	mild atypia (n=5)	moderate atypia (n=11)	severe atypia (n=4)	all (n=20)
Size (mm)	2.4±1.1	2.6±1.3	6.8±3.9	3.4±2.5

p< 0.01*
p<0.05*

Mean ± SD
* Student's *t*- test

Table 3. Ki-67 Labelling Index and AgNORs Score per Nucleus of AAH Lesions

	Normal Peripheral lung (n=20)	AAH				Carcinoma (n=8)
		mild atypia (n=5)	moderate atypia (n=11)	severe atypia (n=4)	all (n=20)	
Ki-67 Labelling Index (%)	0.9±0.9	2.1±0.4	2.2±1.9	2.3±1.1	2.2±1.4	8.2±6.4
			p<0.01*			p<0.01*
				p<0.01*		
AgNORs Score	1.2±0.1	1.4±0.1	1.5±0.1	1.5±0.2	1.5±0.1	3.9±1.1
			p<0.01*			p<0.01*
			p<0.01*			

Mean ± SD
* Student's t- test

Ki-67 labelling index and AgNORs score (Table 3)

The average Ki-67 labelling index of all AAH lesions was 2.2±1.4% (mean ± SD) (Fig. 2A). This result was between those of normal alveolar cells (0.9±0.9%) and corresponding carcinoma cells (8.2 ± 6.4%), and was significantly different from them (p<0.01, Student's t-test). There was no significant correlation between the average Ki-67 labelling index and the degree of atypia of AAH. On the other hand, a statistical correlation between the Ki-67 labelling index and the size of AAH lesion could be detected. (p<0.05, Spearman's correlation coefficient) (data not shown).

The average AgNORs score for all AAH lesions was 1.5 ± 0.1 (Fig. 2B), which was significantly different from that of the normal alveolar cells (1.2 ± 0.1, p<0.01, Student's t-test), and the corresponding carcinoma cells (3.9 ± 1.1, p<0.01, Student's t-test). There was no significant correlation between the average AgNORs score and the degree of atypia of AAH. Furthermore, the statistical correlation between the AgNORs score and the size of AAH lesion could not be detected.

The Kruskal-Wallis test also indicated significant differences between the Ki-67 labelling indices and AgNORs scores of the normal alveolar cells, AAH lesions and carcinoma cells.

Expression of p53 (Table 4)

The expression of p53 in the AAH lesions is shown in Table 4 (Fig. 3). The number of p53-positive AAH lesions for each degree of atypia was: mild, 0 out of 5, moderate, 4 out of 11, and severe, 3 out of 4. All of these lesions showed less than 10% overexpressed nuclei. In the carcinomas, 7 out of 8 cases were p53 positive. The intensity of p53 immunoreactivity in the AAH lesions was weaker than that in the corresponding carcino-
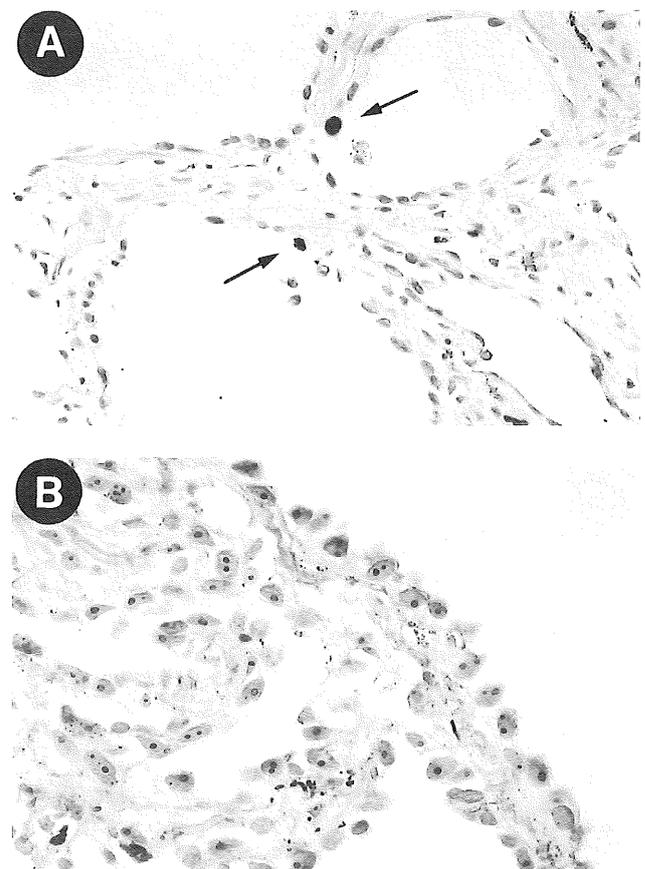
Fig. 2. Ki-67 Immunohistochemistry and AgNORs stain in AAH with moderate atypia (Lesion 6)
(A) There are a few cells showing Ki-67 positive (arrows). (Labelling Index 3.7%) (Original magnification ×100.)
(B) AgNORs stain (score 1.6) (Original magnification ×200.)

mas. Although the expression of p53 had a tendency to reveal a higher rate of positive lesions as the size increased, a statistical correlation could not be detected (Fisher's exact test).

Table 4. Expression of p53 in AAH Lesions

	mild atypia (n=5)	moderate atypia (n=11)	severe atypia (n=4)	all (n=20)	Carcinoma (n=8)
− *	5	7	1	13	1
+ †	0	4	3	7	3
++ ‡	0	0	0	0	2
+++ §	0	0	0	0	2

(with header AAH spanning mild/moderate/severe/all)

* − negative; † + positive cells less than 10%
‡ ++ positive cells less than 20% and 10% or more
§ +++ positive cells 20% or more
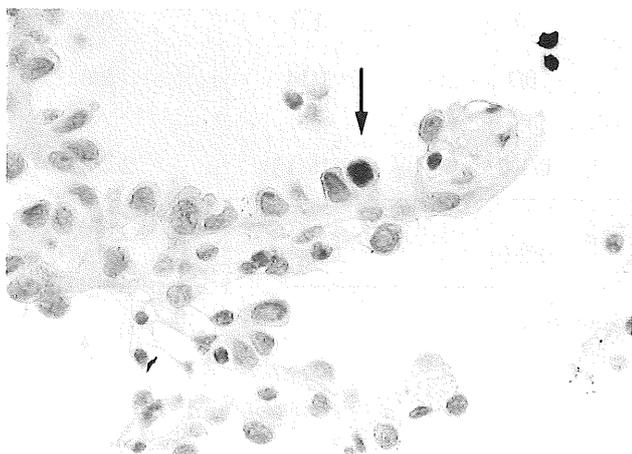

Fig. 3. p53 Immunohistochemistry in AAH with severe atypia (Lesion 2) (Original magnification × 200.)
There is only one cell showing p53 positive (arrow).

Genetic abnormalities of AAH (Table 5)

The genetic alterations detected in the cells of AAH lesions are shown in Table 5. On 3p, we detected LOH of one or more microsatellite markers in 3 out of 17 informative cases. However, 4 out of 6 corresponding carcinomas (informative cases) showed LOH ($p<0.05$, Fisher's exact test). On 9p, 2 out of 15 informative cases showed LOH of one or more microsatellite markers. In carcinomas, 2 out of 4 informative cases showed LOH (not significant, Fisher's exact test). At D9S171 locus (9p), all cases were not informative. At TP53 locus (17p), 1 out of 18 informative lesions showed LOH. In carcinomas, 1 out of 6 informative cases showed LOH (not significant, Fisher's exact test). Interestingly, LOH on 3p, 9p or 17p was limited to the lesions with severe or moderate atypia.

Lesion 1 showed LOH on both 3p (3p21.3, locus marker D3S663) and 17p (Fig. 4A and B), but the other AAH lesions showed LOH on only one chromosome, 3p or 9p. Lesions 16 and 17 were AAH lesions observed in the same case (patient 6), and the pattern of LOH on 9p in each lesion was different (lesion 16 showed LOH on the IFNA locus, whereas lesion 17 showed LOH on the D9S144 locus) (Fig. 5A and B). Other lesions showed the same pattern of LOH as in the corresponding carcinomas. A statistical correlation between the incidence of LOH positive lesions and the size of AAH lesion could not be detected (Fisher's exact test).

DISCUSSION

In human cancer, multistep carcinogenesis theory was advocated by the study of morphological and genetic analysis. For example, a number of colorectal carcinomas appear to arise from adenomas with an accumulation of genetic alterations[32]. This phenomenon is known as adenoma-carcinoma sequence.

In lung cancer, the existence of preneoplastic lesions has also been recognized. In squamous cell carcinoma of the lung, it has been suggested that "dysplasia" in the tracheo-bronchial mucosa might be a preneoplastic lesion. This speculation was supported by the observation of a p53 protein accumulation in the "dysplasia" [1], the presence of genetic alterations including p53 mutation, and the presence of loss of heterozygosity on some chromosomal loci including 3p, 9p and 17p[29, 30] (Nishisaka et al, in press). These findings indicate that a selective genetic change including p53 gene and other genes might lead to clonal expansion of dysplastic cells, and that the accumulation of these genetic alterations may result in a malignant potential.

By contrast, the preneoplastic lesion of lung adenocarcinoma is not fully understood. AAH is a lesion which is small in size and consists of Clara cells or type II alveolar cells[19, 20]. Although this structure is similar to that of well differentiated papillary adenocarcinoma without scar formation, cellular or structural atypia in the AAH lesion is not so severe, and in morphometrical analysis, the mean nuclear area (MNA) and the standard deviation (SD) of the MNA are lower in AAH than in adenocarcinoma[14]. On the other hand, an AAH lesion is frequently found in a lung with adenocarcinoma, independently or close to the adenocarcinoma lesion[15]. Recently, it has been suggested from morphological studies that AAH is a preneoplastic lesion of lung adenocarcinoma. For example, it was reported that some AAH lesions showed aneuploidy[21], abnormal expression of carcinoembryonic antigen (CEA)[20] and p53 nuclear accumulation[11].

In the present study, we attempted to determine the biological characteristics of AAH lesions. As a first step, we evaluated the proliferative activity of AAH lesions using Ki-67 and AgNORs staining. Ki-67 is a nuclear antigen that is only observed in cells in the growth cycle[3], and it is thought that

Table 5. LOH on 3p, 9p and 17p in AAH Lesions

| Microsatellite marker (Locus)	AAH				Carcinoma (n=8)
	mild atypia (n=5)	moderate atypia (n=11)	severe atypia (n=4)	all (n=20)	
3p *D3S2* (3p21)	0/5	1/7	1/1	2/13	2/2
D3S643 (3p21.3)	0	0	0/1	0/1	0/1
D3S663 (3p21.3)	0/5	1/9	0/1	1/15	2/4
D3S1228 (3p14.1- 14.3)	0/4	0/9	0	0/13	0/3
3p Total (%)	0/5(0)	2/10(20)	1/2(50)	3/17*(18)	4/6(67)
9p *IFNA* (9p21- 22)	0	1/3	0	1/3	0/2
D9S144 (9p22)	0/5	1/8	0/1	1/14	2/3
9p Total (%)	0/5(0)	2/9(22)	0/1(0)	2/15(13)	2/4(50)
17p *TP53* (%) (17p13.1)	0/5(0)	1/11(9)	0/2(0)	1/18(6)	1/6(17)

* Significantly different from carcinoma at p<0.05 (by Fisher's exact test)

AgNORs staining detects argyrophilic proteins in DNA loops coding ribosomal RNA, particularly RNA polymerase I and C23[23,35]. As previously reported[11], AAH lesions showed a proliferative activity intermediate between that of normal alveolar cells and carcinoma cells, and this finding indicates two important facts; first, that AAH has specific activity, suggesting that AAH lesions differ from malignant lesions, and second, that AAH could be a preneoplastic lesion of adenocarcinoma. The preneoplastic lesion of squamous cell carcinoma, that is, dysplasia of the lung, also has a proliferative activity intermediate between those of squamous cell carcinoma and metaplastic epithelium (Nishisaka et al, in press). Furthermore, we detected a significant statistical correlation between the size of AAH lesions and Ki-67 labelling indices. However, there was no significant statistical correlation between the proliferative activity and atypia of AAH. Further study on a larger number of AAH lesions might be necessary to ascertain the correlation between proliferative activity and grade of atypia.

In the present series, some AAH lesions showed a weak expression of p53, while normal alveolar cells showed no expression. p53 immunohistochemistry is a useful tool for evaluating cancer cells because abnormal p53 expression indicates a high frequency of abnormalities of the p53 gene in human cancers[1,7]. However, the preliminary data of our PCR-SSCP (single strand conformation polymorphism) analysis of exons 5 through 8 of the p53 gene in the present AAH lesions did not detect any shifted band. Therefore, the abnormal expression in AAH lesions may be an overexpression of the wild type p53 protein induced by genotoxic damage[5]. On the other hand, among three carcinoma lesions with weakly positive expression of p53, in which less than 10% of carcinoma cells were positive, one lesion showed mutation in exon 6 of the p53 gene by means of PCR-SSCP analysis. Therefore, we need to categorize the positive cells with less than 10% of p53 expression as "weakly or mildly positive". We suggest that lesions showing weakly positive p53 expression do not always indicate the existence of a mutation of the gene and that there is a need to confirm the presence of genetic abnormalities by means of molecular biological methods, including PCR-SSCP analysis and direct sequencing.

Several reports have shown genetic changes in AAH. Recently, Westra et al[34] reported that K-ras mutation was observed in 39% of AAH lesions. Ohshima et al[24]. reported that 50% of AAH lesions showed K-ras mutation using PCR-RFLP analysis. We tried to detect genetic alterations in the AAH lesions using microsatellite analysis on 3p, 9p and 17p. The microsatellite markers in this study were located close to some known tumor suppressor genes, that is, the CDKN2 gene on 9p[12], the p53
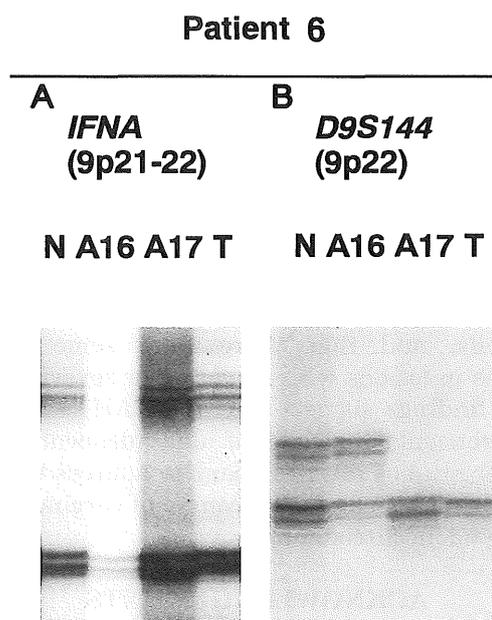
Patient 1

A   B
D3S663   *TP53*
(3p21.3)   (17p13.1)

N A1 T   N   A1   T
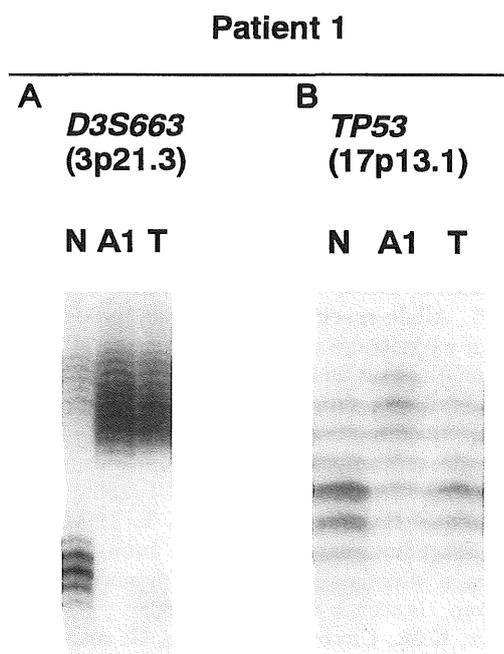
N: normal peripheral lung tissue
A1: AAH lesion 1
T: adenocarcinoma

Fig. 4. LOH on 3p and 17p in AAH with moderate atypia (Lesion 1, patient 1)
(A) AAH lesion 1 (A1) and corresponding adenocarcinoma (T) show the same LOH pattern on 3p21.3 (*D3S663*).
(B) AAH lesion 1 (A1) shows LOH pattern on 17p13.1 (*TP53*), but the corresponding adenocarcinoma (T) retains heterozygosity.

Patient 6

A   B
IFNA   *D9S144*
(9p21-22)   (9p22)

N A16 A17 T   N A16 A17 T

N: normal peripheral lung tissue
A16, 17: AAH lesion 16 and 17
T: adenocarcinoma

Fig. 5. LOH on 9p in AAH with moderate atypia (Lesion 16 and 17, patient 6)
(A) AAH lesion 16 (A16) shows LOH pattern on 9p21-22 (*IFNA*), but lesion 17 (A17) and corresponding adenocarcinoma (T) retain heterozygosity .
(B) AAH lesion 17 (A17) and corresponding adenocarcinoma (T) show the same LOH pattern on 9p22 (*D9S144*), but lesion 16 (A16) retains heterozygosity.

gene on 17p[10] and putative tumor suppressor genes[8] on 3p. Our observations showed 3p, 9p and 17p microsatellite instabilities and that the frequency of LOH in AAH lesions was lower than in adenocarcinomas[26]. In addition, genetic changes were observed only in lesions with severe or moderate atypia. Kishimoto et al[12] also demonstrated a high frequency of genetic abnormalities, including LOH on 3p and 9p in hyperplastic lesions of the bronchus, bronchiole and peripheral lung tissue[8]. However, the relationship between atypia and genetic change has not been reported so far. These findings indicate that activation of oncogenes and inactivation of tumor suppressor genes may induce AAH, and support the idea that AAH is preneoplastic.

Although malignant tumors, including adenocarcinoma of the lung, undergo many genetic changes by multistep carcinogenesis, we found that AAH lesion 1 (patient 1) with moderate atypia had LOH on both 3p and 17p. This result indicates that some AAH lesions may undergo several genetic changes and can be in the late stage of

multistep carcinogenesis. Kishimoto et al[8,12] reported that some hyperplastic lesions had LOH both on 3p and 9p.

In the aerodigestive organs and head and neck tumors, chronic exposure to carcinogens may induce multiple synchronous and metachronous primary tumors at different sites which have the potential to develop into invasive tumors (the "field cancerization" theory)[28]. In the present study, patient 6 had 2 AAH lesions near the primary adenocarcinoma, which had different patterns of LOH on 9p. This result indicates that these synchronous lesions can be induced independently, even though all of the AAH lesions in the other cases showed the same LOH pattern as their corresponding carcinomas. This also indicates that the microsatellite markers used in the present study are not always able to differentiate between multiple primary tumors and intrapulmonary metastasis.

Some AAH lesions (that is in *D3S663* locus in AAH lesion 1, and in *D9S144* locus in AAH lesion

17) showed complete LOH in the microsatellite analysis, the pattern suggesting complete loss of the paternal band or the maternal band. These results suggest the monoclonality of AAH lesions. However, clonal analysis based on X chromosome inactivation is necessary in female cases to confirm this monoclonality.

In conclusion, we demonstrated that the proliferative activity of AAH lesions was intermediate between that of normal alveolar cells and carcinoma cells, and that there were some genetic changes in lesions with moderate or severe atypia. These findings suggest that the AAH lesion may be a preneoplastic lesion of lung adenocarcinoma. Further studies are necessary to address the timing of AAH lesions in the course of carcinogenesis of lung adenocarcinoma.

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