

# Loss of Heterozygosity on Chromosomes 9q and 16p in Atypical Adenomatous Hyperplasia Concomitant with Adenocarcinoma of the Lung

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**Atypical adenomatous hyperplasia (AAH) has recently been implicated as a precursor to lung adenocarcinoma. We previously reported loss of heterozygosity (LOH) in tuberous sclerosis (TSC) gene-associated regions to frequently be observed in lung adenocarcinoma with multiple AAHs. In this study, we analyzed LOH in four microsatellite loci on 9q, including the *TSC1* gene-associated region, and four loci on 16p, including the *TSC2* gene-associated region, in both 18 AAHs and 17 concomitant lung adenocarcinomas from 11 patients. Seven of 18 (39%) AAHs and 9 of 17 (53%) adenocarcinomas displayed LOH on 9q. Five (28%) AAHs and seven (41%) adenocarcinomas harbored LOH at loci adjacent to the *TSC1* gene. Four of 18 (22%) AAHs and 6 of 17 (35%) adenocarcinomas displayed LOH on 16p. One (6%) AAH and five (29%) adenocarcinomas harbored LOH at loci adjacent to the *TSC2* gene. These findings may indicate a causal relationship of LOH on 9q and 16p in a fraction of AAH lesions and adenocarcinomas of the lung. Especially, the frequencies of LOH on 9q and at the *TSC1* gene-associated region were high. The *TSC1* gene or another neighboring tumor suppressor gene on 9q might be involved in an early stage of the pathogenesis of lung adenocarcinoma. (*Am J Pathol* 2001, 159:1941–1948)**

Carcinogenesis is a multistep process that results from an accumulation of genetic alterations in oncogenes and tumor suppressor genes. It is reasonable to regard each preneoplastic lesion as possibly having a characteristic genetic change and it is essential to investigate the biological features of preneoplastic lesions to elucidate the pathogenesis of carcinomas. In lung cancers, squamous

dysplasia has long been recognized as a preneoplastic lesion of squamous cell carcinoma.<sup>1,2</sup> However, the etiology of adenocarcinoma, one of the major histological types of lung cancer, is not well understood. Several genetic alterations in atypical adenomatous hyperplasia (AAH), such as *K-ras* or *p53* mutations or loss of heterozygosity (LOH) on chromosomes 3p, 9p, or 17p, have been reported.<sup>3–10</sup> These genetic abnormalities and other immunohistochemical or morphometric abnormalities in AAH overlap with those of adenocarcinomas.<sup>11–13</sup> Thus, AAH has been implicated as a preneoplastic lesion of lung adenocarcinoma, and listed as a precursor lesion in the World Health Organization 1999 classification of lung tumors.<sup>14</sup>

Tuberous sclerosis (TSC) is a relatively common autosomal-dominant disease that causes mental retardation, seizures, and multiple hamartomas in many organs including the brain, eyes, kidney, skin, and heart. Mutations of either the *TSC1* or the *TSC2* gene are responsible for this disease.<sup>15,16</sup> The lesion most commonly described in the lung is lymphangioleiomyomatosis, which occurs in 1% of patients with TSC and affects only females.<sup>17</sup> Multifocal micronodular pneumocyte hyperplasia (MMPH) has also been described as a rare pulmonary manifestation of TSC.<sup>18</sup> MMPH is so similar to AAH morphologically that histological distinction between MMPH and AAH is difficult.<sup>19</sup> We previously reported that LOH in the *TSC* gene-associated regions was frequently observed in lung adenocarcinoma with multiple AAHs.<sup>20</sup> In this study, we analyzed microsatellite alterations at several microsatellite loci including the *TSC* gene-associated regions in both AAH lesions and concomitant adenocarcinomas to clarify the stage of lung adenocarcinoma pathogenesis in which these genetic alterations are involved.

## Materials and Methods

From November 1997 through June 1998, 126 patients underwent surgical resection of lung cancer at our hos-

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**Table 1.** Clinicopathological Characteristics of Patients

Patient	Age	Gender	Smoking (pack-years)	Past history of malignancy	Family history of malignancy	Number of adenocarcinomas	Number of AAH
1	74	M	41	–	–	4	2
2	72	F	0	–	+	1	1
3	60	F	0	+	(lung, sister) <sup>†</sup>	4	3
4	51	F	0	–	+	1	3
5	47	M	4	–	–	1	2
6	62	F	0	–	–	3	6
7	75	F	0	–	–	1	1
8	65	M	45	–	–	1	2
9	65	M	70	+	–	1	5
10	61	F	0	–	–	1	2
11	69	F	0	–	+	1	2
				(stomach)	(stomach, father)		

\*Organs affected by a past malignancy.

<sup>†</sup>Affected organ and family member with history of malignancy.

pital. AAH was found in 22 patients with adenocarcinoma, 2 patients with adenosquamous carcinoma, 1 with squamous cell carcinoma, and 1 with large cell carcinoma. AAH is much more frequently found in patients with adenocarcinoma than in those with other histological subtypes.

We analyzed LOH on chromosomes 9q and 16p in 18 AAHs and 17 adenocarcinomas from 11 patients. The patients' characteristics are summarized in Table 1. There were four males and seven females, and their ages ranged from 47 to 74 years. Seven of the 11 (64%) patients were non-smokers. Two patients had a past history of malignancy. Three patients had a family history of malignancy in first-degree relatives; two were lung cancers, one a gastric cancer. There were three patients with multiple adenocarcinomas, and nine with multiple AAHs. All patients with multiple adenocarcinomas had concomitant multiple AAHs.

All resected specimens prefixed with 100% methanol were sliced at 5-mm intervals and examined macroscopically. Appropriate tissue sections were fixed with 100% methanol and embedded in paraffin. It is difficult to extract enough amount of DNA for molecular analysis from a tiny lesion, such as AAH. DNA extracted from formalin-fixed materials is often fragmented artificially. Using methanol fixation, even relatively higher molecular weight DNA was preserved well.<sup>21</sup>

Primary lung adenocarcinomas and AAHs were evaluated microscopically by conventional hematoxylin and eosin (H&E) staining. The pathological characteristics of the 17 adenocarcinomas and 18 AAHs were assessed by two pathologists (AO and TY).

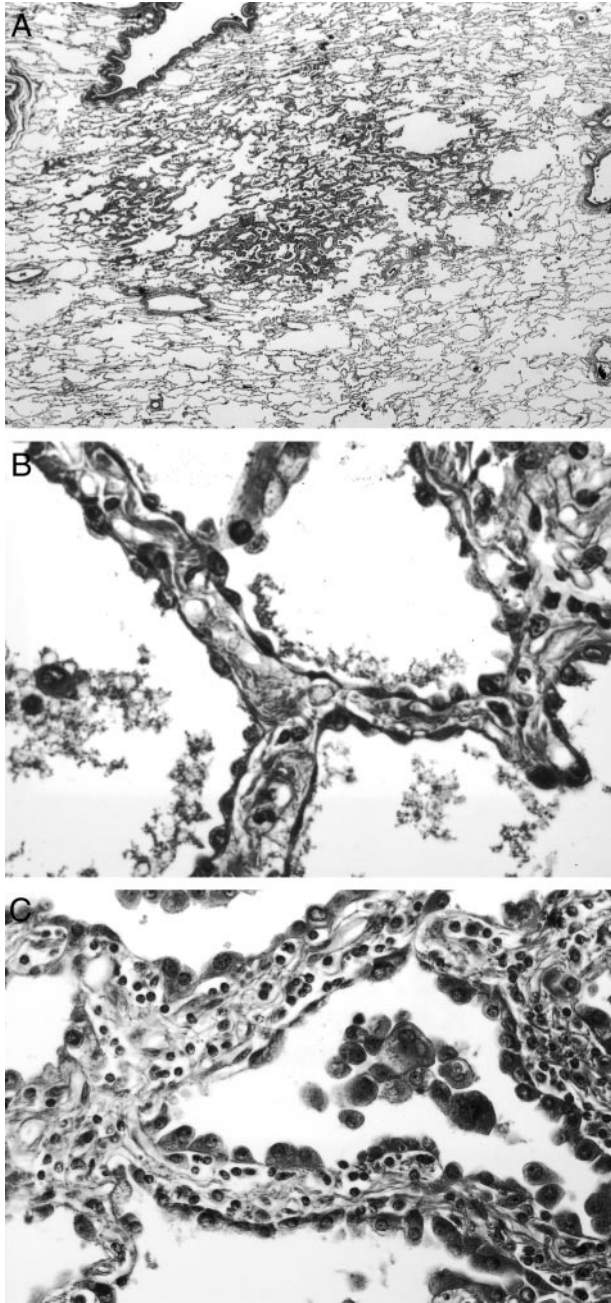
### Histological Evaluation of Adenocarcinoma

Histological typing of adenocarcinomas was performed according to the World Health Organization classification of lung tumors.<sup>14</sup> Nuclear atypia was categorized into three grades: 1, nuclei that were uniform in size and slightly larger than those of reactive type II alveolar epi-

thelial cells; 2, nuclei that were in uniform size and up to twice the size of reactive type II alveolar epithelial cells; 3, nuclei of various sizes and more than twice the size of reactive type II alveolar epithelial cells. The mitotic indices were divided into three grades: 1,  $\leq 5$  mitotic cells/10 high-power fields; 2, 6 to 15/10 high-power fields; 3,  $\geq 16/10$  high-power fields. The scar grades were classified into four grades based on fibrotic foci in the tumors: 1, no or minimal desmoplasia; 2, fibroblastic tissue with a small amount of collagen; 3, fibroblastic tissue with moderate to abundant collagen; 4, hyalinized tissue.<sup>22</sup>

### Histological Criteria of AAH

We analyzed solitary AAHs in this study, AAHs continuous with or directly adjacent to a primary adenocarcinoma were not included. The histological diagnosis of AAH was based on the following criteria, as previously described.<sup>23</sup> 1) The lesion had well-defined boundaries and consisted of proliferation of single-layered atypical epithelial cells without central scar formation or collapse (Figure 1A). 2) The cytoplasm was eosinophilic, and the cells often had a rounded or domed appearance resembling either type II pneumocytes or Clara cells. 3) The atypical cells in AAH had usually hyperchromatic nuclei and inconspicuous nucleoli, but the atypia was less marked than that of adenocarcinoma cells. AAHs were classified into two grades:<sup>24</sup> 1) Low-grade AAH; the cell density was low to moderate, and the cells were arranged in a single layer, intermittently or focally and continuously, on the alveolar septa (Figure 1B). Their nuclei were mostly small, but occasionally large, and exhibited lesser degrees of variation in size, shape, and hyperchromasia than high-grade AAH. 2) High-grade AAH; the cells were continuously and densely arranged in a single layer, but did not exhibit the piled-up structure often observed in adenocarcinoma (Figure 1C). Their nuclei showed significant atypia, but lacked the margin irregularity and eosinophilic nucleoli observed in frank adenocarcinoma.



**Figure 1.** Histological features of AAH (H&E staining). **A:** The lesion has well-defined boundaries and no central scar formation or collapse. **B:** Low-grade AAH: the cell density is low to moderate, with the cells arranged in a single layer, intermittently or focally and continuously, on the alveolar septa. **C:** High-grade AAH: the cells are continuously and densely arranged in a single layer, but do not exhibit the piled-up structure often observed in adenocarcinoma. Original magnifications:  $\times 2$  (A),  $\times 150$  (B),  $\times 100$  (C).

### Microdissection and DNA Extraction

Serial 5- $\mu\text{m}$  slices were made from each block for microdissection. The slides were dewaxed with xylene and rehydrated in a graded alcohol series, then stained with H&E to define the location of AAHs or adenocarcinomas. Microdissection was performed under a microscope (BX50W1; Olympus, Tokyo, Japan) by using a microcapillary tube drawn to a thin tip with a micropipette puller

(PC-10; Narishige, Tokyo, Japan) and a joystick-operated hydraulic micromanipulator (ONO-125; Olympus-Narishige, Tokyo, Japan). The microdissected cells were allowed to adhere to Parafilm (American Can, Greenwich, CT) and placed in 500- $\mu\text{l}$  microcentrifuge tubes. Normal lymph node tissue or normal lung tissue was scraped with a 27-gauge needle to provide a normal control. DNA was extracted with a DNA extractor WB kit (Wako Pure Chemicals, Osaka, Japan). The DNA concentration was adjusted to  $\sim 50$  cell equivalents per  $\mu\text{l}$ .

### Polymerase Chain Reaction (PCR)-Based LOH Analysis

We analyzed LOH on chromosome 9q (including the *TSC1* gene-associated region) and 16p (including the *TSC2* gene-associated region), using the following microsatellite markers. Four markers on 9q from centromere to telomere, *D9S146* (9q13), *D9S149* (9q34), *D9S150* (9q34), and *DBH* (9q34);<sup>25</sup> and four markers on 16p from centromere to telomere, *D16S300* (16p11.1-11.2), *D16S292* (16p13.12-13.13), *D16S291* (16p13.1), and *D16S525* (16p13.3).<sup>26,27</sup> PCR was performed in a 20- $\mu\text{l}$  volume of a mixture containing 10 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 1.0 to 1.5 mmol/L  $\text{MgCl}_2$ , 200  $\mu\text{mol/L}$  of each Cy 5'-end labeled primer (Pharmacia Biotech, Tokyo, Japan), 0.25 U of *Taq* polymerase (TaKaRa Biomedicals, Shiga, Japan) and then cycled 36 to 38 times in a GeneAmp PCR System 9600 (Perkin Elmer, Foster City, CA); each cycle consisted of 1 minute at 94°C for denaturation, 2 minutes at 55 to 62°C for annealing, 1 minute at 72°C for strand elongation, and 7 minutes at 72°C for final elongation. The PCR products were diluted with a loading buffer consisting of 95% formamide, 20 mmol/L ethylenediaminetetraacetic acid (pH 8.0), and Dextran blue, and denatured for 5 minutes at 98°C. The samples were electrophoresed on 5% polyacrylamide gels containing 8.3 mol/L urea for 3 hours at 34 W using an ALFred DNA sequencer (Pharmacia Biotech). To confirm the reproducibility of the experiment, all cases were examined at least twice by independent PCR and electrophoresis. LOH was considered to be present if the reduction rate of the height of the allele in the tumor was  $>40\%$ , as previously defined.<sup>28</sup>

### Statistical Analyses

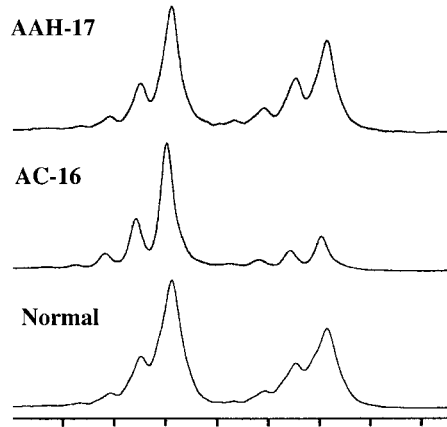
The two-sided Fisher's exact test was used for statistical analysis (Stat View-J 5.0, Macintosh). A *P* value  $<0.05$  was considered significant.

### Results

We examined 18 AAHs and 17 adenocarcinomas of the lung from 11 patients for LOH on chromosome 9q, including the *TSC1* gene-associated region (9q34), and chromosome 16p, including the *TSC2* gene-associated region (16p13.3), by using two sets of four microsatellite markers, respectively. Representative examples are

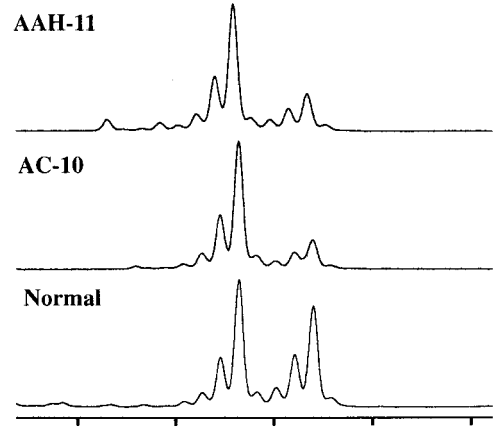
**A. Patient-10**

*D9S146*



**B. Patient-6**

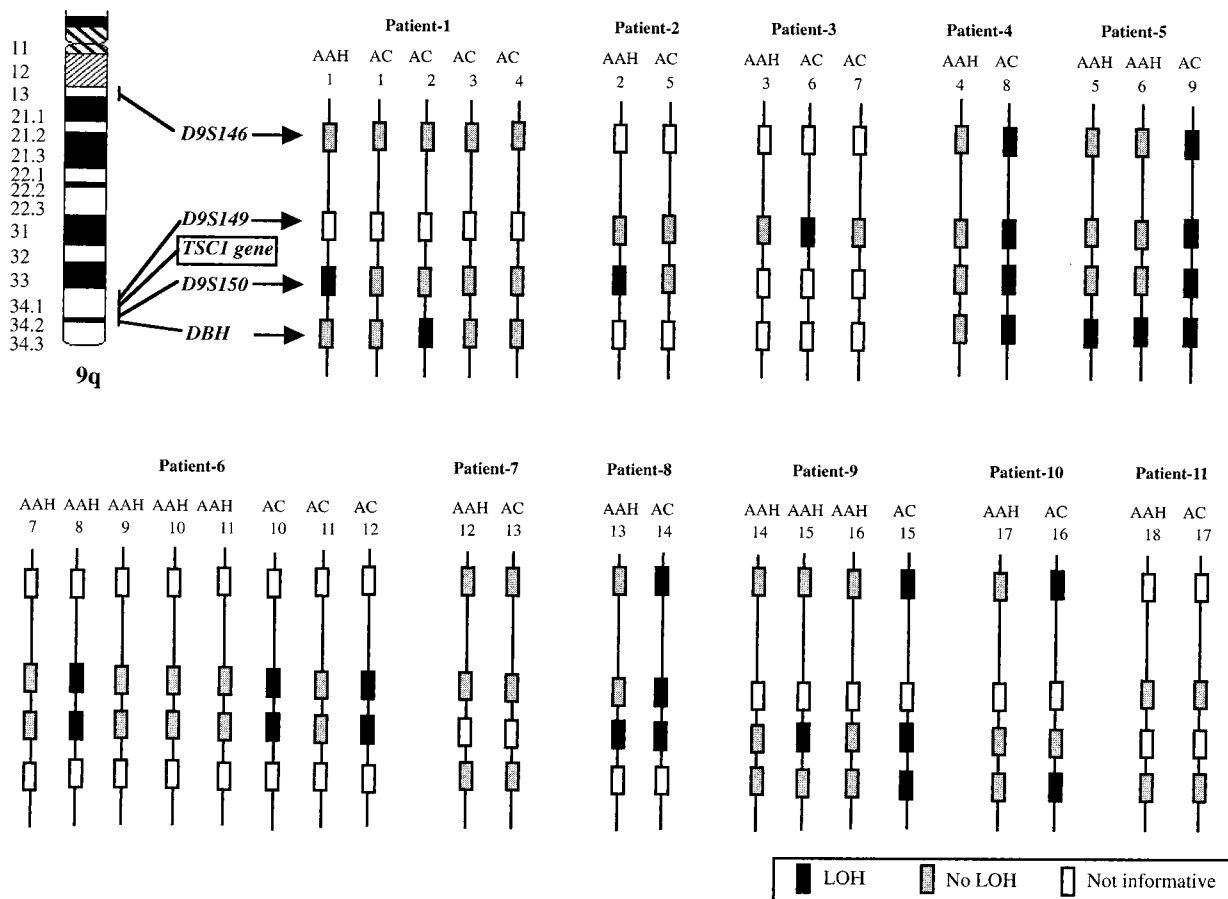
*D16S291*



**Figure 2.** Representative examples of microsatellite analyses of chromosomes 9q and 16p in AAHs and adenocarcinomas (AC). **A:** AAH-17 retained heterozygosity at *D9S146*. AC-16 showed LOH at *D9S146*. **B:** Both AAH-11 and AC-10 showed LOH at *D16S291*.

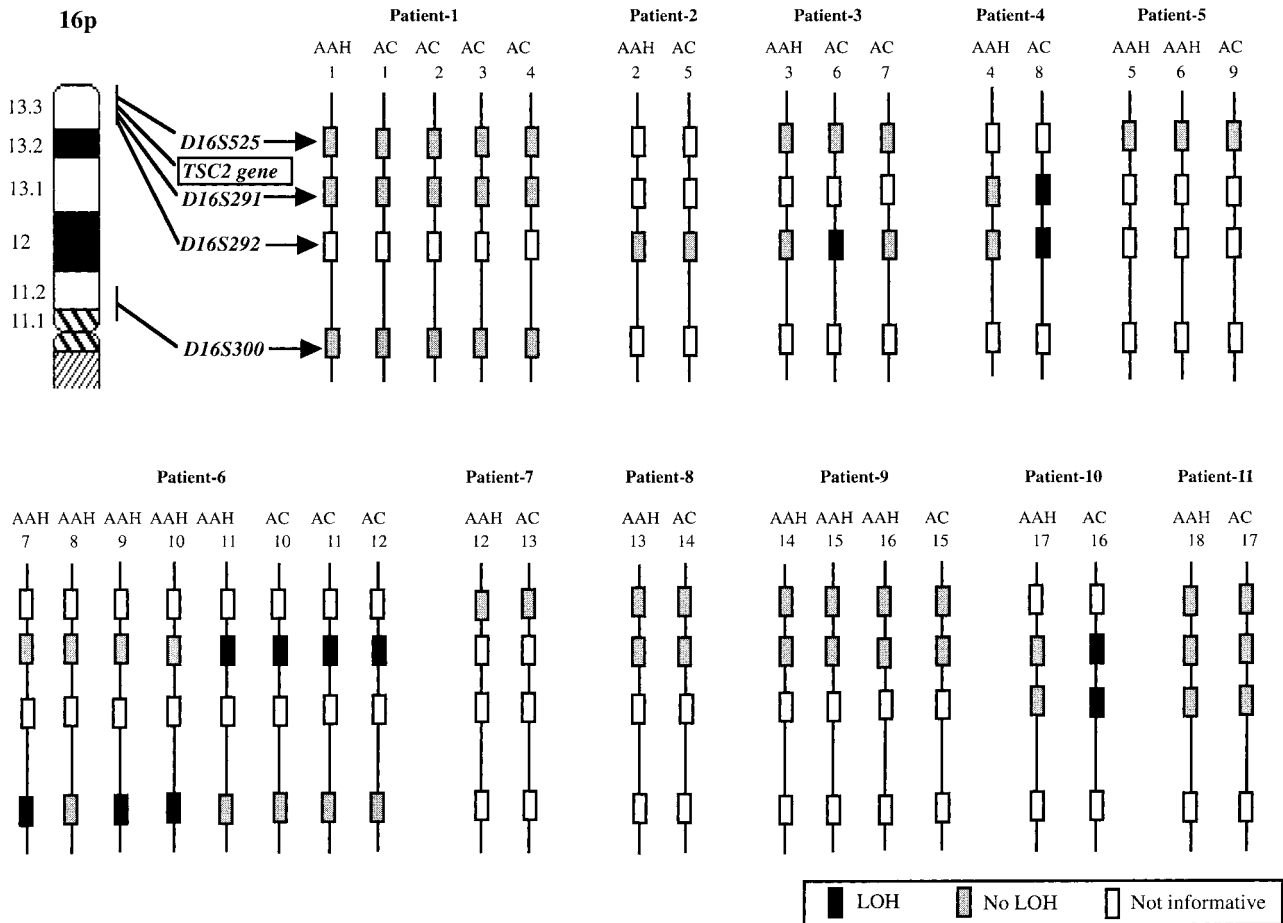
shown in Figure 2. The distributions of 9q region deletions in AAHs and adenocarcinomas are shown in Figure 3. Seven of 18 AAHs (39%) and 9 of 17 adenocarcinomas (53%) showed LOH at one or more loci on chromosome

9q. Seven of nine adenocarcinomas with LOH on 9q (78%) harbored LOH at all informative loci on 9q (complete LOH). However, only one AAH showed complete LOH on 9q. Five of seven AAHs with LOH on 9q harbored



**Figure 3.** The distributions of deletions on 9q in AAHs and adenocarcinomas. AC, primary lung adenocarcinoma.





**Figure 4.** The distributions of deletions on 16q in AAHs and adenocarcinomas. AC, primary lung adenocarcinoma.

LOH at the same locus as the concomitant adenocarcinomas.

The distributions of deletions on 16p in AAHs and adenocarcinomas are shown in Figure 4. Four of 18 AAHs (22%) and 6 of 17 adenocarcinomas (35%) showed LOH at one or more loci on chromosome 16p. These four AAHs were from the same patient (patient 6). Two of six adenocarcinomas with LOH on 16p (33%) showed complete LOH on 16p, but all AAHs harbored LOH at only one locus among all of the informative loci. One AAH harbored LOH at the same locus as the concomitant adenocarcinomas.

The *TSC1* gene is located between *D9S149* and *D9S150*. Five of seven AAHs with LOH on 9q (71%) and seven of nine adenocarcinomas with LOH on 9q (78%) showed LOH at loci adjacent to the *TSC1* gene, ie, loci including *D9S149* or *D9S150*. The *TSC2* gene is located between *D16S291* and *D16S525*. None of the AAHs or adenocarcinomas harbored LOH at *D16S525*. One of four AAHs with LOH on 16p (25%) and five of six adenocarcinomas with LOH on 16p (83%) showed LOH at loci adjacent to the *TSC2* gene, ie, at *D16S291*.

The frequencies of LOH at all loci examined were as follows: 5 of 20 informative samples (25%) at *D9S146*, 7 of 29 (29%) at *D9S149*, 11 of 28 (39%) at *D9S150*, 7 of 20 (35%) at *DBH*, 3 of 13 (23%) at *D16S300*, 3 of 11 (27%)

at *D16S292*, 6 of 25 (24%) at *D16S29*, and none (0%) at *D16S525*.

We analyzed the relationships between LOH on chromosome 9q or 16p, or LOH at microsatellite loci adjacent to the *TSC1* or *TSC2* gene, and the following histological characteristics of adenocarcinoma and AAH: size, histological subtype (bronchioloalveolar carcinoma versus other subtypes), vascular invasion, lymphatic permeation, nuclear atypia, mitotic index, or scar grade of adenocarcinoma, and the size or histological grade of AAH. No histological characteristic was found to be associated with LOH on 9q or 16p, or with LOH at microsatellite loci adjacent to the *TSC1* or *TSC2* gene (Tables 2 and 3).

### Discussion

Little is known about the etiology of lung adenocarcinoma, as compared with squamous cell carcinoma. AAH has recently been implicated as a preneoplastic lesion of lung adenocarcinoma. Miller<sup>29</sup> initially proposed a pulmonary adenoma-carcinoma sequence analogous to that of the colon. AAH is much more frequently associated with adenocarcinoma than other histological subtypes of lung cancer.<sup>30</sup> Multiple AAHs are occasionally detected

**Table 2.** Relationships between Histological Characteristics of Primary Adenocarcinoma and LOH on Chromosomes 9q and 16p

	<i>n</i>	LOH on 9q	LOH on 16p	TSC1LOH	TSC2LOH
Tumor size					
≤2 cm	9	4	3	3	2
>2 cm	8	5 (0.64)*	3 (1.0)	4 (0.64)	3 (0.62)
Histological subtype					
BAC	8	4	2	3	1
Other than BAC	9	5 (1.0)	4 (0.62)	4 (1.0)	4 (0.29)
Vascular invasion					
Negative	10	5	4	4	3
Positive	7	4 (1.0)	2 (1.0)	3 (1.0)	2 (1.0)
Lymphatic permeation					
Negative	11	5	3	4	2
Positive	6	4 (0.62)	3 (0.60)	3 (0.64)	3 (0.28)
Nuclear atypia					
1 or 2	6	2	1	1	0
3	11	7 (0.34)	5 (0.33)	6 (0.30)	5 (0.10)
Mitotic index					
1	12	5	5	4	4
2 or 3	5	4 (0.29)	1 (0.60)	3 (0.59)	1 (1.0)
Scar grade					
1 or 2	9	4	3	3	2
3 or 4	8	5 (0.64)	3 (1.0)	4 (0.64)	3 (0.62)

BAC, bronchioloalveolar carcinoma.  
 \*Numbers in parentheses are *P* values (Fisher's exact test).

in patients with multiple lung adenocarcinomas.<sup>29</sup> In our series, the histological subtype was adenocarcinoma in 22 of 26 (85%) lung cancer patients with AAH, and all patients with multiple adenocarcinomas had concomitant multiple AAHs. These findings suggest that AAH and adenocarcinoma might be induced by common etiologic factors.

AAH has been reported to have several genetic alterations in common with lung adenocarcinoma. Mutations of *K-ras* codon 12 have variously been detected in 15 to 50% of AAHs and 22 to 42% of adenocarcinomas.<sup>7-10</sup> LOH on chromosome 3p was detected in 10 to 18% of AAHs and 12 to 67% of adenocarcinomas.<sup>4-6</sup> LOH on chromosome 9p was detected in 5 to 13% of AAHs and 19 to 50% of adenocarcinomas.<sup>4-6</sup> We demonstrated AAHs to harbor LOH on chromosomes 9q and 16p, LOHs that are also found in lung adenocarcinomas. The frequency of LOH on 9q, especially in the *TSC1* gene associated regions, was high. Petersen and colleagues<sup>31</sup> reported LOH on 9q34 to be significantly associated with lung adenocarcinoma. Our results were also consistent with this observation. The relatively high frequency of LOH on 9q34 in AAH lesions and adenocarcinomas suggests that a novel tumor suppressor gene for lung ade-

nocarcinomas may exist in this region, and the *TSC1* gene is one candidate.

Hung and colleagues<sup>3</sup> and Kishimoto and colleagues<sup>4</sup> detected more frequent and extensive 3p and 9p losses in carcinomas than in corresponding preneoplastic lesions, and the identical allele was lost among them, an allele-specific loss. Their findings were consistent with our results. Six of seven AAHs with LOH on 9q and all AAHs with LOH on 16p showed LOH at only one microsatellite locus. However, the allelic losses on 9q and 16p were more extensive in adenocarcinomas than in AAHs. In particular, the rate of complete LOH on 9q was high (78%) in adenocarcinomas. This progressive loss on 9q during progression might suggest existence of more than one tumor suppressor gene on 9q for lung adenocarcinoma. In addition, the identical allele was lost among multiple AAHs and concomitant adenocarcinomas in the same patients. These observations indicate that one allele might be inactivated congenitally because of mutation or epigenetically, and the remaining allelic loss would then be acquired. This suggests a possible role of genetic predisposition in the pathogenesis of lung adenocarcinoma with AAH. In our series, 7 of 11 (64%) patients with lung ade-

**Table 3.** Relationships between Histological Characteristics of AAH and LOH on Chromosomes 9q and 16p

	<i>n</i>	LOH on 9q	LOH on 16p	TSC1LOH	TSC2LOH
Size					
<5 mm	10	6	1	4	0
≥5 mm	8	1 (0.07)*	3 (0.27)	1 (0.31)	1 (0.44)
Grade					
Low	10	4	2	4	0
High	8	3 (1.0)	2 (1.0)	1 (0.31)	1 (0.44)

Low, low-grade AAH; high, high-grade AAH.  
 \*Numbers in parentheses are *P* values (Fisher's exact test).

carcinoma and corresponding AAH were non-smokers and 5 of 11 (45%) had either a past or family history of malignancy. These findings might also indicate genetic predisposition.

Muir and colleagues<sup>18</sup> reported that MMPH is distinguishable from AAH by the following features: AAH cells have a greater degree of nuclear-to-cytoplasmic ratio than MMPH, less well circumscribed with peripheral lepidic spread, less prominent interstitial reticulin, and fewer air space macrophages than MMPH. However, some investigators have reported that MMPH is so similar to AAH morphologically that histological distinction between MMPH and AAH is difficult.<sup>19</sup> Thus, we supposed that these two conditions might share some molecular mechanisms of pathogenesis. Genetic alteration in *TSC1* gene-associated regions is a candidate.

If we postulate that the *TSC1* gene itself is responsible for early development of lung adenocarcinoma, there would be an obvious discrepancy. We would more frequently encounter TSC patients with MMPH. However, MMPH is believed to be a rare pulmonary manifestation of TSC, and only a few such cases have been reported.<sup>18,19</sup> How can we explain this discrepancy? Because MMPH is a subtle pulmonary lesion, underestimation of the incidence of MMPH might be one explanation. The life expectancy of patients with TSC is relatively short,<sup>32</sup> and this is another possible reason. We do not understand how many genetic alterations are required for formations of MMPH and AAH. If several genetic alterations are required, low incidence of MMPH association in TSC patients might be explained. This is the third possible reason. The forth and most probable explanation is that *TSC1* itself is not the responsible gene but rather that some novel tumor suppressor gene for lung adenocarcinoma exists very close to the *TSC1* gene.

In our present study, we analyzed only a limited number of lesions. Further study of a large number of adenocarcinomas and AAH lesions is clearly required. We did not analyze LOH in AAH adjacent to adenocarcinoma, because it was difficult to convincingly differentiate the border between AAH and adenocarcinoma. If we could analyze several adenocarcinomas obviously associated with AAH adjacently, it would become more convincing. In addition, an extensive mutation analysis or demonstrating the suppressed expression of the *TSC1* gene in lung adenocarcinoma might be helpful in answering the above question.

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