Clinicopathological and Molecular Evidence Indicating the Independence of Bronchioloalveolar Components from Other Subtypes of Human Peripheral Lung Adenocarcinoma

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ABSTRACT

Although human lung adenocarcinoma has diverse histological subtypes, the correlation between histological subtypes and occurrence of the *p53* gene mutation has been given less attention. We investigated 145 surgically resected lung adenocarcinomas to search for the incidence of *p53* mutations and for record data on survival in each histological subtype, according to the new WHO criteria (1999). The frequency of *p53* mutation in bronchioloalveolar carcinoma (BAC; 0% in 17 cases) and BAC with invasive growth component (BAC-invasive; 11% in 27 cases), which is conventionally categorized as the mixed subtype in WHO typing, were apparently significantly lower than in other types (non-BAC including acinar, papillary, solid, or mixed histology with these subtypes; 48% in 101 cases; $P < 0.01$). Multivariate analysis revealed that the histological subtype including BAC-invasive was a strong, independent, and significant prognostic factor ($P < 0.03$), as were tumor size and pathological stage ($P < 0.001$ and 0.002, respectively) for overall survival. However, the occurrence of *p53* mutation itself was seen to be significant only in case of the univariate analysis. Therefore, histological subtyping may be a better prognostic indicator than is *p53* mutation. These findings suggest that the WHO classification with the BAC and BAC-invasive from other histological subtypes may prove useful to predict the outcome for surgically treated patients with lung adenocarcinoma.

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INTRODUCTION

Lung carcinomas are currently the leading cause of cancer-related death in most countries, including Japan. Among non-small cell lung cancers, adenocarcinoma is increasing in frequency and accounts for almost half the number of lung cancers (1). Adenocarcinoma is of diverse subtypes (1, 2); however, little information is available regarding biological characteristics of each subtype. BAC[2], a subcategory of lung adenocarcinoma, has a fairly good prognosis for surgically treated patients; however, the prognosis of BAC with fibrotic foci associated with destructive and invasive growth (BAC-invasive), which is categorized in mixed subtypes in the WHO classification published in 1999, is poorer than that of BAC without it (2). Because emerging evidence suggests that accumulation of allelic mutations largely affects the biological and clinical behaviors of neoplasms, this means that histological subtypes may respond to the diverse gene abnormalities. However, there is a paucity in data on the relationship between histological subtypes of human peripheral lung adenocarcinoma and genetic aberrations.

Neoplastic transformation is considered to be the result of a multistep accumulation of genetic abnormalities, including either activation of oncogenes or inactivation of tumor suppressor genes. *p53* tumor suppressor gene mutation is common and frequent among genetic abnormalities in various human cancers, suggesting that the occurrence is a fundamentally important step in carcinogenesis, and it may even play a key role in the clinical prognosis. Regarding non-small cell lung cancers, although many workers have investigated the relationship of p53 abnormalities and prognosis, the results have differed; hence, the clinicopathological significance of p53 alteration has remained unknown. The findings conflicted perhaps because of the methodology used in examinations, such as immunohistochemistry and genomic analysis, regardless of histological subtypes. An important issue to be considered is that there is little information regarding if and when *p53* mutation occurs during tumor progression. Although recent studies (3–7) suggest that BAC likely derives from an atypical adenomatous hyperplasia, a putative premalignant lesion, we find no documentation as to when *p53* mutation occurs.

In the present study, we examined (*a*) the frequency of *p53* mutation and (*b*) the clinicopathological background in each histological subtype, classified according both to the current WHO criteria, and the minor modification, including a proposed

[2] The abbreviations used are: BAC, bronchioloalveolar carcinoma; SSCP, single-strand conformation polymorphism.

Table 1 Relationship between p53 mutations, clinical characteristics, and histological subtypes of lung tumors

Characteristics	No. of examined	No. of cases (%)		*P*	*r*
		Wild type	Mutated		
Total	145	94 (65)	51 (35)		
Age (yr; mean ± SD)		67 ± 9	65 ± 10	0.21[a]	
Sex					
Female	63	46 (73)	17 (27)	0.07[b]	
Male	82	48 (59)	34 (41)		
Tumor size (mm; mean ± SD)		29 ± 12	32 ± 17	0.19[a]	
Pathological stage					
I	82	56 (68)	26 (32)	0.32[b]	
II–IV	63	38 (60)	25 (40)		
Smoking					
Nonsmoker	60	46 (77)	14 (23)	0.012[b]	0.22[c]
Smoker	85	48 (56)	37 (44)		
Histological subtype					
BAC	17	17 (100)	0 (0)	<0.01[b]	
BAC-invasive	27	24 (89)	3 (11)		
Acinar	4	3 (75)	1 (25)		
Papillary	59	32 (54)	27 (46)		
Solid	38	18 (47)	20 (53)		

[a] Student's *t* test.
[b] χ^2 test.
[c] Spearman's rank correlation coefficient.

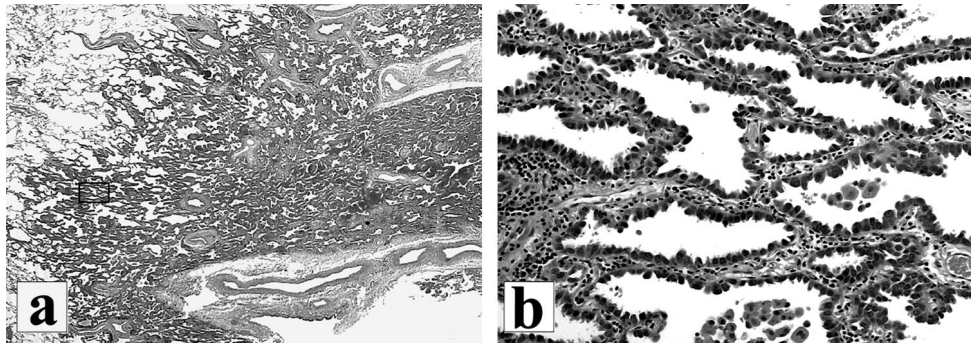

Fig. 1 Histological features of BAC. A good demarcation between cancer and noncancerous lesion is apparent (*a*; H&E; original magnification ×12.5). Cancer cells proliferate along the mildly fibrotic alveolar septa (*b*; H&E; original magnification ×200).

subgroup of BAC-invasive. On the basis of our data, we propose that BAC-invasive type should be classed independently from the mixed subtype of lung adenocarcinoma, with regard to both *p53* mutations and clinical prognosis. We also found that histopathological subtyping with this modification is a more productive and independent prognostic indicator than is *p53* mutation.

MATERIALS AND METHODS

Patients and Tissue Preparation. *p53* mutational analysis was made on 145 specimens with primary lung adenocarcinomas. The patients were surgically treated at Kyushu University Hospital, Fukuoka, Japan during the period from 1995 to 1998. These Japanese patients were never treated with chemotherapy or irradiation before the surgery. Table 1 shows the clinicopathological and histopathological background of these patients. The average age was 66.5 ± 9.5 years, and the man/woman ratio was 1.3. According to the tumor-node-metastasis staging system of the Union International Contre le Cancer (8), 82 patients were in pathological stage I, 12 were in stage II, 47 were in stage III, and 4 were in stage IV. Patients with a smoking history were divided into smokers including not only current smokers but also those with a past history of smoking or nonsmokers without any past history of smoking. The resected specimens were fixed with 10% buffered formalin and sliced at 5 mm. Sections (4 μm thick) obtained from the maximal cut surface area of the cancer tissue were stained with H&E, elastica Van Gieson's stain, and Alcian blue.

Histological Classification. The histopathological classification was essentially done based on the criteria of WHO (Ref. 1; Figs. 1–3) but with some modifications, as indicated below, and was determined by four pathologists (T. K., S. M., Y. M., K. Sue.). An adenocarcinoma showing a pure bronchioloalveolar growth pattern and neither evidence of stromal, vascular, nor pleural invasion is categorized in BAC according to the classification of WHO (Fig. 1). Concerning adenocarcinoma of mixed subtypes, a predominant subtype was evident in this study (Fig. 2 and 3). An adenocarcinoma, predominantly with a bronchioloalveolar component but with stromal invasion, was classified as an independent group, *i.e.*, BAC with invasive

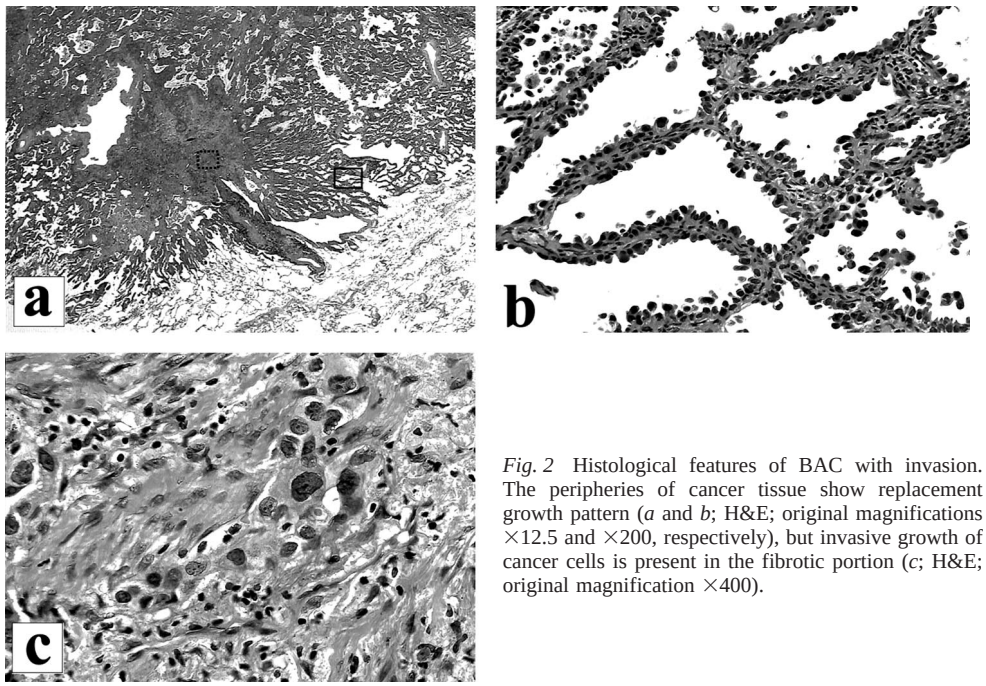

Fig. 2 Histological features of BAC with invasion. The peripheries of cancer tissue show replacement growth pattern (*a* and *b*; H&E; original magnifications ×12.5 and ×200, respectively), but invasive growth of cancer cells is present in the fibrotic portion (*c*; H&E; original magnification ×400).

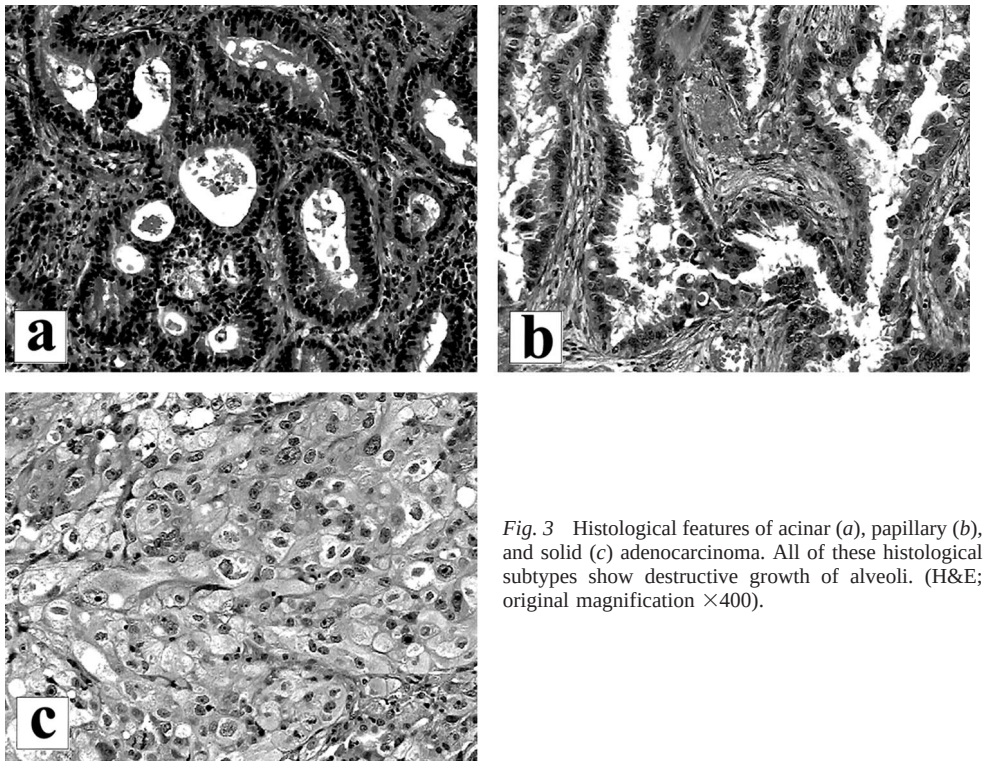

Fig. 3 Histological features of acinar (*a*), papillary (*b*), and solid (*c*) adenocarcinoma. All of these histological subtypes show destructive growth of alveoli. (H&E; original magnification ×400).

growth (Fig. 2). In this category, we defined the area of fibrosis with invasive growth as less than 50% of the tumor area. The invasive growth of cancer cells was confirmed using elastica Van Gieson's stain.

DNA Extraction and PCR of Exon 2 to 9 of the *p53* Gene. The 4-μm-thick sections, excised from the paraffin-embedded blocks of the resected lung cancers, were dewaxed and lightly stained with hematoxylin. The cancer cells, dissected

Table 2 Sequence of the primers for nested and semi-nested PCR methods

Region	Codon	PCR step		Sequence	bp
2	1–25	1st PCR	Sense	CTGGATCCCCACTTTTCCTC	150
			Antisense	CTTCCAATGGATCCACTCAC	
		2nd PCR	Sense	ATCCCCACTTTTCCTCTTGC	146
3	26–32	1st PCR	Sense	AGCGAAAATTCATGGGACTG	152
			Antisense	TGGGTGAAAAGAGCAGTCAG	
		2nd PCR	Antisense	AAAAGAGCAGTCAGAGGACCA	146
4	33–93	1st PCR	Sense	CCTCTGACTGCTCTTTTCAC	215
			Antisense	CAGGGGCCAGGAGGGGGCTG	
		2nd PCR	Sense	TGACTGCTCTTTTCACCCAT	211
	94–125	1st PCR	Sense	CACCAGCAGCTCCTACACCG	192
			Antisense	CATTGAAGTCTCATGGAAG	
		2nd PCR	Antisense	TGAAGTCTCATGGAAGCCAG	189
	126–163	1st PCR	Sense	ACTCTGTCTCCTTCCTCTTC	146
			Antisense	GCTTGTAGATGGCCATGGCG	
		2nd PCR	Antisense	TAGATGGCCATGGCGCGGAC	141
	146–186	1st PCR	Sense	GCTGTGGGTTGATTCCACAC	167
			Antisense	AACCAGCCCTGTCGTCTCTC	
		2nd PCR	Sense	TGGGTTGATTCCACACCCCC	163
6	187–224	1st PCR	Sense	GCCTCTGATTCCTCACTGATT	175
			Antisense	TCCTCCCAGAGACCCCAGTT	
		2nd PCR	Sense	CTGATTCCTCACTGATTGCTC	165
			Antisense	CAGAGACCCCAGTTGCAAAC	
7	225–261	1st PCR	Sense	CCTCATCTTGGGCCTGTGTT	171
			Antisense	CAGTGTGCAGGGTGGCAAGT	
		2nd PCR	Sense	CTTGGGCCTGTGTTATCTCC	161
			Antisense	GTGCAGGGTGGCAAGTGGCT	
8	262–306	1st PCR	Sense	TTCCTTACTGCCTCTTGCTT	206
			Antisense	CACCGCTTCTTGTCCTGCTT	
		2nd PCR	Sense	TGCCTCTTGCTTCTCTTTTC	198
9	307–331	1st PCR	Sense	CAGTTATGCCTCAGATTCAC	152
			Antisense	TGATAAGAGGTCCCAAGACT	
		2nd PCR	Sense	CACCTTTCCTTGCCTCTTTC	126

under a light microscope, were collected into 0.6-ml siliconized microcentrifuge tubes. The precipitates were digested with 0.02% proteinase K in 200 μl of 20 mM Tris-HCl (pH8.0) containing 1 mM EDTA and 0.5% Tween 20 at 37°C for 42 h and then were heated at 95°C for 15 min to inactivate proteinase K activity. One μl of digested sample was then used for the following PCR reactions.

Exons 2–9 of the *p53* gene were analyzed using PCR-SSCP and *p53*-specific oligonucleotide primers (Table 2). Thereafter, semi-nested or nested PCR was done as described (9), but with some modifications. The first PCR reaction was performed in a 20-μl reaction mixture containing 20 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of deoxyribonucleotide triphosphate, 0.2 μM of outer primer pairs, 1 unit of Taq DNA polymerase (Takara Shuzo Co., Kyoto, Japan), and 1 μl of template DNA. The first PCR product was diluted to a ratio of 1:50 in distilled water, and 1 μl of the dilution, as a template, was applied to the second PCR. The PCR reaction conditions for the first PCR were 95°C for 20 s, annealing temperature 59–60°C for 20 s, and 72°C for 20 s (35 cycles), whereas the second PCR was run at 95°C for 20 s, 59°C for 20 s, and 72°C for 20 s (30 cycles). A Perkin-Elmer 9600 Thermal Cycler (Perkin-Elmer Co., Norwalk, CT) was used.

SSCP Analysis. Mutations in exons 2 through 9 of the *p53* gene were identified by SSCP, as described (9), but with minor modifications. Each secondary PCR product (0.6 μl) was mixed with 4.5 μl of loading buffer composed of 83% formamide containing 8.3 mM EDTA and 0.05% methyl violet. After denaturing DNA at 80°C for 5 min, 2 μl of the mixture was electrophoresed at 800 v for 2 h in a 5% acrylamide gel with 5% glycerol. PCR-SSCP procedures were repeated at least twice to confirm the reproducibility for the same mobility shift of the bands.

DNA Sequencing. All of the mutations detected by PCR-SSCP were confirmed by direct DNA sequencing, as described (9), but with minor modifications. Any band showing any aberrant mobility shifts was excised from the gel and eluted into water at 80°C for 5 min, followed by reamplification with the same inner primers and conditions, as described above. The samples were subjected to subsequent direct DNA sequencing, using a Thermo Sequenase core sequencing kit (Amersham, CA) and SQ-5500 DNA sequencer (Hitachi, Japan).

Immunohistochemical Analysis of P53 Protein. An immunohistochemical study was done using monoclonal anti-human P53 antibody (DO7; Novocastra, Newcastle, United Kingdom). For the antigenic retrieval of the antibody, the sections were autoclaved for 5 min at 121°C in 0.1 M citrate buffer solution (pH 6.0). After treating the sections with 1.5% milk solution to reduce the nonspecific absorption of antibody, the sections were reacted with the primary monoclonal antibody diluted to 1:100 overnight at 4°C. The tissue sections were treated with biotin-labeled antimouse antibody and then with 0.1% H_2O_2-methanol solution, followed by the streptavidin-biotin-peroxidase complex method (10).

Table 3 Summary of p53 mutation and clinicopathological data

Case	Histological subtype[a]	p Stage	Exon	Codon	Nucleotide change	Amino acid change	Protein expression[b]
1	B-inv	Ia	5	138	GCC to GTC	Ala to Val	+
2	B-inv	Ia	4	46	TCC to TCT	Silent	−
3	B-inv	Ia	7	240	AGT to AAT	Ser to Asn	+
4	A	IIb	5	136	CAA to CA	Frameshift	−
5	P	Ia	8	280	AGA to GGA	Arg to Gly	+
6	P	Ia	9	316	CCC to CCA	Pro to His	−
7	P	Ia	5	170	ACG to ATG	Thr to Met	2+
				172	GTT to GTG	Val to Met	
8	P	Ia	5	139	AAG to AAA	Silent	2+
9	P	Ia	4	122	GTG to GTA	Silent	−
10	P	Ia	5	175	CGC to GGC	Arg to Gly	2+
11	P	Ia	5	154	GGC to AGC	Gly to Ser	−
12	P	Ia	7	248	CGG to CTG	Arg to Leu	2+
13	P	Ia	6	210	AAC to AC	Frameshift	−
14	P	Ia	5	159	GCC to GTC	Ala to Val	2+
15	P	Ia	4	72	CGC to CCC	Arg to Pro	−
16	P	Ia	5	135	TGC to TGG	Cys to Trp	2+
17	P	Ib	5	179	CAT to GAT	His to Asp	2+
18	P	Ib	8	280	AGA to GGA	Arg to Gly	−
19	P	IIb	7	245	GGC to GAC	Gly to Asp	2+
20	P	IIb	5	178	CAC to AAC	His to Asn	2+
21	P	IIIa	8	272	GTG to ATG	Val to Met	2+
22	P	IIIa	4	69	GCT to GT	Frameshift	−
23	P	IIIa	7	241	TCC to TTC	Ser to Phe	2+
24	P	IIIa	7	248	CGG to TGG	Arg to Trp	2+
25	P	IIIa	5	144	CAG to TAG	Gln to Stop	−
26	P	IIIa	8	291	AAG to TAG	Lys to Stop	−
27	P	IIIb	6	193	CAT to CTT	His to Leup	2+
28	P	IIIb	8	292	AAA to AGA	Lys to Arg	−
29	P	IIIb	5	126	ACT to CCT	Thr to Pro	+
				127	TCC to TTC	Ser to Phe	
30	P	Ia	5	155	ACC to ATG	Thr to Met	−
31	P	Ia	5	146	TGG to TAG	Trp to Stop	−
32	S	Ia	5	135	TGC to TAC	Cys to Tyr	2+
33	S	Ia	4	60	CCA to CTA	Pro to Leu	2+
34	S	Ia	7	245	GGC to TGC	Gly to Cys	+
35	S	Ia	5	161	GCC to GAC	Ala to Asp	+
36	S	Ib	5	175	CGC to GGC	Arg to Gly	−
37	S	Ib	5	132	AAG to AAC	Lys to Asn	2+
38	S	Ib	8	267	CGG to TGG	Arg to Trp	2+
39	S	Ib	6	213	CGA to TGA	Arg to Stop	−
40	S	IIb	6	220	TAT to TGT	Tyr to Cys	−
41	S	IIIa	8	267	CGG to CGA	Silent	2+
42	S	IIIa	7	248	CGG to CAG	Arg to Gln	2+
43	S	IIIa	5	158	CGC to CTC	Arg to Leu	2+
44	S	IIIa	5	157	GTC to GGC	Val to Gly	2+
45	S	IIIa	5	130	CTC to CGC	Leu to Arg	2+
46	S	IIIa	5	132	AAG to AAT	Lys to Asn	+
47	S	IIIa	5	146	TGG to TAG	Trp to Stop	−
48	S	IIIa	5	155	ACC to ATC	Thr to Ile	2+
49	S	IIIa	5	158	CGC to CTC	Arg to Leu	−
50	S	IIIb	7	249	AGG to AGT	Arg to Ser	+
51	S	IV	5	166	TCA to TGA	Ser to Stop	−

[a] B-inv, bronchioloalveolar carcinoma with invasive foci; A, acinar; P, papillary; S, solid.
[b] 2+, 50% positive cells; +, <50% positive cells; −, <10% positive cells.

The P53-labeling index of cancer cells in each cancer tissue was determined by counting the number of P53-positive cells among at least 300 cancer cells.

Statistical Analysis. To estimate the correlation between the frequency of *p53* mutation and clinicopathological data, including histological subtypes, tumor size, smoking status, and pathological stage, χ^2 test, Student's *t* test, and the Mann-Whitney *U* test were used. All of the *P*s were based on two-hypothesis testing, and statistical significance was assumed at a level of $P < 0.05$. Survival curves were obtained using the Kaplan-Meier method, and the statistical significance of differences was calculated using the log-rank test. Multivariate analysis was performed to identify independent prognostic factors and to assess the hazard ratio with the Cox proportional hazards model, using the Statistical Package for Social Science (SAS). In this model, seven factors potentially related to survival (age

Table 4 Correlation between p53 mutation and p53 overexpression in relation to histological subtype

Histological subtypes	No. examined	p53 mutation	p53 overexpression (%) +	p53 overexpression (%) −	*P*	Concordance rate (%)
Total	145	+	32 (60)	19 (40)	<0.001[a]	67
		−	29 (30)	65 (70)		
BAC	17	+	0 (0)	0 (0)		94
		−	1 (6)	16 (94)		
BAC-invasive	27	+	2 (67)	1 (33)	<0.05[b]	85
		−	3 (13)	21 (87)		
Subtotal		+	2 (67)	1 (33)	<0.002[a]	89
		−	4 (10)	37 (90)		
Acinar	4	+	0 (0)	1 (100)	0.75[b]	50
		−	1 (33)	2 (67)		
Papillary	59	+	16 (59)	11 (41)	0.34[a]	57
		−	15 (47)	17 (53)		
Solid	38	+	14 (70)	6 (30)	0.20[a]	58
		−	9 (50)	9 (50)		
Subtotal		+	30 (63)	18 (37)	0.12[a]	57
		−	25 (47)	28 (53)		

[a] χ^2 test.
[b] Fisher's exact probability test.

at surgery, gender, histological subtype, *p53* gene status, tumor size, smoking history, and pathological stage) were included, and the model selection for identifying the subset of significant variables was based on the stepwise method for background selection. Discriminant analysis was also examined between an independent prognostic factor obtained from multivariate analysis and other factors. In this analysis, a stepwise method for background selection was used.

RESULTS

Histopathological Findings. The 145 cases consisted of 17 nonmucinous and mucinous BACs, 27 BACs with invasive growth (Figs. 1 and 2, respectively; Table 1), 4 acinar adenocarcinomas, 59 papillary adenocarcinomas, and 38 solid adenocarcinomas (Fig. 3, *a–c*, respectively; Table 1). A mean of the area of lepidic growth in BAC with invasion was 90.4 ± 10.4% (mean ± SD).

The Frequency of *p53* Mutation in Each Histological Subtype. Of 145 cases, 51 (35%) had a mutation in the *p53* gene in exons 2–9 (Tables 1 and 3), 5 (9.8%) in exon 4, 26 (51%) in exon 5, 4 (7.8%) in exon 6, 8 (15.7%) in exon 7, 7 (13.7%) in exon 8, and 1 (2%) in exon 9. Cases 7 and 29 had two different mutations (Table 3). No mutation was found in exons 2 and 3. Regarding the relation of *p53* mutation to histological subtypes, *p53* mutations were found in 3 of 27 (11%) BACs with invasion, 1 (25%) of acinar adenocarcinomas, 27 (46%) of papillary adenocarcinomas, and 20 (53%) of solid adenocarcinomas (Table 1), but no mutation was found in the case of BAC alone ($P < 0.01$; Table 1).

Relationship between *p53* Mutation and Clinicopathological Data. There was no difference in mean age, mean tumor size, and pathological stage with regard to *p53* gene status (Table 1). The frequency of *p53* mutation was significantly higher in smokers than that in nonsmokers, and the correlation coefficient was apparently significant ($P = 0.012$; $r = 0.22$; Table 1). There was a tendency toward a more frequent occurrence of *p53* mutation in men than of that in women but with no statistical significance ($P = 0.07$; Table 1).

Immunohistochemical Overexpression of P53 Protein and the Relation to *p53* Gene Mutation. In Table 4, 61 of the 145 cases (42%) showed overexpression of P53 protein. The concordance rate of overall cases between P53 immunohistochemistry and *p53* gene status was 67% with a statistical significance ($P < 0.001$). However, the concordance rates were higher in BAC without and with invasion (94% and 85%, respectively; $P < 0.05$) than that of the acinar, papillary, and solid adenocarcinomas ranging from 50 to 58%.

Relationship between Histological Subtypes and Smoking History and *p53* Mutational Pattern. The frequency of smoking in non-BAC cases was significantly higher than that of BACs and BACs with invasion (71% and 30%, respectively; $P < 0.001$). G:C → T:A transversions were observed in 9 (18.8%) of 48 non-BAC types, but in none of 3 BACs with invasion.

Survival Rate, Histological Subtype, and *p53* Gene Status. Analyses for the relationship between histological subtypes, *p53* gene status, and survival rates revealed that all of the patients with BAC with invasion, acinar, papillary, or solid type had a poorer prognosis than in cases with BAC alone, which was statistically significant ($P = 0.001$; Fig. 4*A*). We also studied the relationship between *p53* gene status and overall survival. Of the 145 cases, patients with *p53* mutations had a shorter survival period than did those without any *p53* mutation (Fig. 4*B*; $P < 0.05$); however, among patients with either acinar, papillary, or solid type, no statistical significance was noted with regard to *p53* gene status (Fig. 4*C*). Examining the relationship between the type of *p53* mutation and survival by stage, no statistical significance was apparent regarding survival time between patients with *p53* null mutations and missense mutations in any stage (data not shown).

Univariate and Multivariate Analyses for Survival. Univariate analysis of age, gender, histological subtype, *p53* gene status, tumor size, smoking history, and pathological stage for survival significance was made. Tumor size, pathological stage, histological subtype, smoking, and *p53* mutation (as the lower *P*) were significant prognostic factors (Table 5). How-

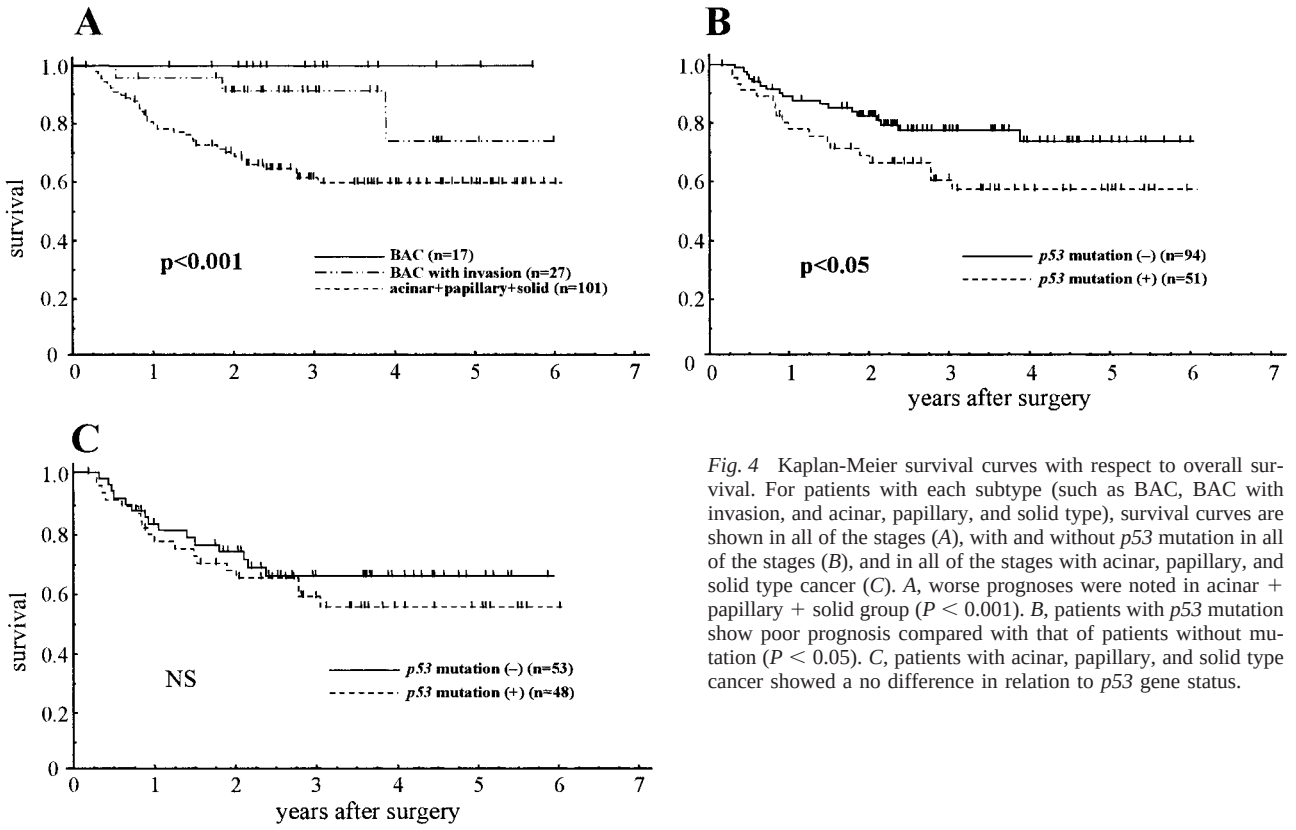

Fig. 4 Kaplan-Meier survival curves with respect to overall survival. For patients with each subtype (such as BAC, BAC with invasion, and acinar, papillary, and solid type), survival curves are shown in all of the stages (*A*), with and without *p53* mutation in all of the stages (*B*), and in all of the stages with acinar, papillary, and solid type cancer (*C*). *A*, worse prognoses were noted in acinar + papillary + solid group ($P < 0.001$). *B*, patients with *p53* mutation show poor prognosis compared with that of patients without mutation ($P < 0.05$). *C*, patients with acinar, papillary, and solid type cancer showed a no difference in relation to *p53* gene status.

Table 5 Analysis for overall survival in all of the patients

	P	Parameter estimate	SE	Hazard ratio (95% CI)[a]
Univariate analysis				
Age (yr; mean ± SD)	NS	NS	NS	NS
Sex	NS	NS	NS	NS
Tumor size (mean ± SD)	0.0001	0.055	0.009	1.06 (1.04~1.08)
Pathological stage	0.0001	0.74	0.16	2.10 (1.54~2.85)
Smoking	0.025	0.86	0.39	2.37 (1.11~5.05)
p53 mutation	0.049	0.66	0.33	1.93 (1.00~3.71)
Histological subtype[b]	0.003	0.48	0.16	1.62 (1.18~2.23)
Multivariate analysis				
Age (yr; mean ± SD)	NS	NS	NS	NS
Sex	NS	NS	NS	NS
Tumor size (mean ± SD)	0.0001	0.048	0.01	1.05 (1.03~1.07)
Pathological stage	0.0011	0.59	0.18	1.80 (1.26~2.55)
Smoking	NS	NS	NS	NS
p53 mutation	NS	NS	NS	NS
Histological subtype[b]	0.028	1.26	0.57	3.53 (1.15~10.8)

[a] CI, confidence interval; NS, not significant.
[b] Histological subtypes were arranged into three groups, such as BAC, BAC-invasive, and non-BAC type.

ever, multivariate analysis of the same variables revealed that tumor size and patients' stage were statistically significant, but smoking and *p53* mutation were not significant (Table 5). In these analyses, histological subtypes were arranged into three categories in accordance to the prognostic ranks. Categories 1, 2, and 3 were BAC alone, BAC with invasion, and combined group with acinar, papillary, and solid type, respectively, and the survival rates of these groups showed a statistical significance in the log-rank test with the Kaplan-Meier method (Fig. 4*A*). In all of the cases, *p53* mutation was not an independent prognostic factor, whereas histological subtype was an independent prognostic factor with a statistical significance ($P = 0.028$).

Discriminant Analysis for Histological Subtype. Univariate and multivariate discriminant analyses were made to determine which factors would significantly contribute to the

Table 6 Univariate analysis of discriminant analysis of histological subtype in all of the patients

	P	λ of Wilks	F-value
Age (yr; mean ± SD)	NS[a]	NS	NS
Sex	0.0001	0.82	7.54
Tumor size (mean ± SD)	0.0001	0.86	5.54
Pathological stage	NS	NS	NS
Smoking	0.0001	0.78	9.97
p53 mutation	0.0001	0.66	8.02

[a] NS, not significant.

Table 7 Multivariate analysis of discriminant analysis for histological subtype in all of the patients

	P	λ of Wilks
Tumor size (mean ± SD)	0.007	0.41
Sex	0.013	0.38
Smoking	0.021	0.35
p53 mutation	0.0001	0.46

histological subtype. *p53* mutation, tumor size, gender, and smoking history were apparently the discriminant factors (Tables 6 and 7).

DISCUSSION

Our study clearly elucidated that: (*a*) the frequency of *p53* gene mutation varies with the histological type of peripheral human adenocarcinomas. This mutation was rare both in BAC (0 of 17 cases; 0%) and BAC-invasive (3 of 27; 11%) but relatively frequent in non-BAC (48 of 101; 48%); and (*b*) the concordance between P53 immunohistochemistry and *p53* gene status is much higher in BAC and BAC-invasive (average, 89%; $P < 0.002$) than that in other cases (average, 57%; $P = 0.12$). To our knowledge, this is the first molecular evidence related to possible biological properties in each histological subtype of human lung adenocarcinoma.

Although a significantly poor prognosis was observed in patients with *p53* mutation, the frequency of *p53* mutation did not significantly affect the prognosis in patients with non-BAC. Furthermore, multivariate analysis for overall survival revealed that *p53* mutation was not a significant prognostic factor, whereas the histological subtype is an independent and significant indicator. Together with the discordance between *p53* mutation and P53 immunoreactivity shown in Table 4, these findings suggest that the enhanced expression of P53 is likely attributable to mechanisms other than *p53* mutation in non-BAC, and there is a lack of significant correlation between *p53* mutation and the prognosis in patients with non-BAC. Inversely, BAC and BAC-invasive showed strong concordance between *p53* mutation and P53 immunoreactivity, which suggests that the prolonged half-life of P53 seemed largely attributable to the mutated *p53* allele.

The discordance between *p53* gene status and P53 immunoreactivity within each subtype of lung adenocarcinoma has been unclear. *In vitro* studies suggested that the type of mutation, including null mutation, other cellular proteins associated with P53, including murine double minute 2 or viral oncoproteins such as simian virus 40 large T antigen, and DNA damage may have an effect.

Many studies (11–13) revealed that *p53* mutations are more frequent in squamous cell carcinoma, well-known smoking-related malignancies, than in adenocarcinomas. In the present study, the frequency of *p53* mutation in papillary and solid adenocarcinoma was relatively higher than in BAC groups and close to findings in the case of the squamous cell carcinoma, where the reported rate was from 60 to 68% (11–13). G:C → T:A transversion in the *p53* gene is a smoking-related mutation (13, 14) and is assumed to arise as a direct consequence of benzopyrene diol epoxide-DNA adducts (15). In the present study, the relationship of smoking history with *p53* mutation in all of the patients showed a correlation coefficient, and the ratio of patients who used tobacco was significantly higher in patients with non-BAC. These results suggest that a history of tobacco smoking was strongly associated with *p53* mutation in these subtypes, similar to findings with squamous cell carcinoma of the lung. Conversely, BAC and BAC-invasive showed a poorer correlation to smoking than did non-BAC.

Histological subtypes reflecting *p53* status are useful prognostic markers for determining survival time after surgical intervention. *p53* mutation was not a significant prognostic factor, yet discriminant analysis revealed that *p53* mutation was a significant factor for discriminating histological subtype.

Our findings also suggest that BAC-invasive should be classed independently from the mixed subtype in the WHO criteria, because this type of adenocarcinoma has a better prognosis than other mixed types composed mainly of non-BAC (Fig. 4*A*). There are reports that showed the size of the scar or the area of lepidic growth component is prognostically important in lung adenocarcinoma (16, 17); however, in the present study, no statistical significance was found between the area of lepidic growth component and patients' survival (data not shown). This conflict may be related to the fact that we did not limit the tumor size of subjects examined in this study, which has been suggested to be an important prognostic factor, within 3 cm in diameter.

In conclusion, BAC is independent from other histological subtypes from the point of clinicopathological and molecular evidence. Evaluation of the histological subtype based on the current WHO classification can predict the clinical prognosis more so than analyzes of the *p53* gene mutation. We propose that patients with non-BAC type adenocarcinoma should be prescribed adjuvant therapies regardless of the *p53* gene status.

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