Different Subtypes of Human Lung Adenocarcinoma Caused by Different Etiological Factors

Evidence from *p53* Mutational Spectra

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Human lung adenocarcinomas are only relatively weakly associated with tobacco smoke, and other etiological factors need to be clarified. These may also vary with the histopathology. Because the *p53* mutation status (frequency and spectrum) of a carcinoma can provide clues to causative agents, we subclassified 113 adenocarcinomas into five cell types: hobnail, columnar/cuboidal, mixed, polygonal, and goblet (54, 23, 18, 13, and 5, respectively) and investigated relationships with *p53* mutations and smoking history. In the hobnail cell type, a low mutational frequency (37%) and a high proportion of transitions (65%), especially G:C to A:T transitions at CpG dinucleotides (45%) associated with spontaneous mutations, were found with a weak relation to tobacco smoke. In contrast, a high mutation frequency (70%) with a higher proportion of transversions (50%), especially G:C to T:A (44%) on the nontranscribed DNA strand, caused by exogenous carcinogenic agents like tobacco smoke, were observed for the columnar cell type, as with squamous cell carcinomas. These results indicate that two major subtypes of lung adenocarcinoma exist, one probably caused by tobacco smoke, and the other possibly due to spontaneous mutations. For the prevention of lung adenocarcinomas, in addition to stopping tobacco smoking, the elucidation of endogenous mechanisms is important. (*Am J Pathol* 2000, 157:2133–2141)

Lung cancer constitutes one of the leading causes of cancer death in the world,^{1,2} and its incidence is increas-

ing in Japan.³ Histologically lung cancer is classified into four major types: squamous cell carcinoma, small cell carcinoma, adenocarcinoma, and large cell carcinoma, based on the 1999 WHO classification.⁴ Of the four types, adenocarcinoma is now the most common, and its proportion is increasing not only in Japan but also in the United States.^{5,6} Therefore, it is necessary to develop new approaches for its prevention, early detection, and treatment. To achievement of this goal, elucidation of etiological factors and carcinogenic mechanisms is important. Exogenous factors, especially tobacco smoke, are established causes of squamous cell and small cell carcinomas, but other, as yet unknown, endogenous factors may be more important for adenocarcinomas.^{6–8} One reason why little is known about their nature may be that adenocarcinomas have generally been analyzed as a discrete group. However, their histopathology is very complicated, and the several subtypes may each have their own etiology.^{4,9,10}

Mutations in the *p53* tumor suppressor gene appear to be important for the genesis of many kinds of tumors, including lung cancers.^{8,11–14} Their frequency and mutational spectra can be said to reflect carcinogenesis by exogenous or endogenous factors and thus may be helpful for identification of the responsible agents.^{8,11–13} With lung cancers, tobacco smoke, one of the most important exogenous carcinogenic agents, has been shown to frequently cause *p53* mutations, especially G:C to T:A transversions.^{8,12,15–17} On the other hand, transitions, especially G:C to A:T transitions at CpG sites, are thought to be caused by endogenous mechanisms involved in spontaneous mutations.^{8,12} Therefore the mutation frequency and spectrum may provide information on the etiological factors for lung cancer.

Working on the hypothesis that different subtypes of adenocarcinoma are caused by different etiological factors, we first subclassified a large series and examined *p53* gene mutations in exons 4–8 and 10. As controls,

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Table 1. *p53* Mutations and Clinicopathological Parameters

	No. of cases (%)					
	Adenocarcinomas		Squamous cell carcinomas		Total	
	Examined	Mutated	Examined	Mutated	Examined	Mutated
All cases	113	46 (41)	38	22 (58)	151	68 (45)
Age at surgery (years)						
Mean $\pm$ SD	60 $\pm$ 11	59 $\pm$ 11	67 $\pm$ 9	67 $\pm$ 9	62 $\pm$ 11	62 $\pm$ 11
Sex						
Male	63	29 (46)	35	20 (57)	98	49 (50)
Female	50	17 (34)	3	2 (67)	53	19 (36)
Location						
Central	0	0	15	10 (67)	15	10 (67)
Peripheral	113	46 (41)	23	12 (52)	136	58 (43)
Differentiation						
Well	43	16 (37)	2	1 (50)	45	17 (38)
Moderately	50	20 (40)	28	17 (61)	78	37 (47)
Poorly	20	10 (50)	8	4 (50)	28	14 (50)
Pathological stage						
IA	43	17 (40)	6	3 (50)*	49	20 (41) [†]
IB	14	3 (21)	8	2 (25)	22	5 (23)
IIA	6	2 (33)	1	1 (100)	7	3 (43)
IIB	1	0 (0)	7	5 (71)	8	5 (63)
IIIA	17	8 (47)	5	4 (80)	22	12 (55)
IIIB	29	14 (48)	11	7 (64)	40	21 (53)
IV	3	2 (67)	0	0	3	2 (67)
Smoking status						
Nonsmoker	50	17 (34)	5	3 (60)	55	20 (36)
Smoker	63	47 (75)	33	19 (58)	96	48 (50)
Adenocarcinoma subtypes						
WHO classification						
Acinar	22	12 (55)				
Papillary	16	5 (31)				
Bronchioloalveolar carcinoma	2	1 (50)				
Solid adenocarcinoma with mucin	5	4 (80)				
Adenocarcinoma with mixed subtypes	68	24 (35)				
Cell type classification						
Hobnail cell type	54	20 (37)				
Columnar cell type	23	16 (70)				
Mixed cell type	18	2 (11)				
Polygonal cell type	13	6 (46)				
Goblet cell type	5	2 (40)				

*Stage I vs. stages II–IV, $P = 0.038$ (by Fisher's exact probability test).

[†]Stage I vs. stages II–IV, $P = 0.022$ (by χ^2 test).

squamous cell carcinomas were also examined. Then the relationships among histological subtypes, *p53* mutational status, and smoking history were assessed.

Materials and Methods

Lung Cancers, Clinicopathological Data, and Smoking Histories

We examined 151 non-small-cell lung carcinomas (113 adenocarcinomas and 38 squamous cell carcinomas) that had been resected consecutively from 1989 to 1993 at Cancer Institute Hospital, Tokyo, Japan. None of the patients had received chemotherapy or radiotherapy before surgery, but 79 patients (60 with adenocarcinoma and 19 with squamous cell carcinoma) underwent postoperative adjuvant therapy. The study population was aged 26–84 (median 62) years and comprised 98 men and 53 women. Data for other clinicopathological parameters, differentiation and location of the tumor, patholog-

ical stages, and patient's smoking status are presented in Table 1. Differentiation of tumors was determined according to the 1999 WHO classification of lung tumors.⁴ The location of a tumor in the lung was classified as central when it was considered to have arisen in a main to segmental bronchus, and peripheral when in a subsegmental or more distal bronchus.¹⁸ The pathological stages (pStages) were determined using the International Union Against Cancer (UICC) TNM staging system,¹⁹ and statistical difference was calculated between two groups, pStage I and pStages II–IV. The patient's smoking history (number of cigarettes per day, starting age, and duration of smoking) was obtained from preoperative personal interviews and expressed as nonsmokers and smokers, the latter including both patients with a past history of smoking and current smokers.

Histopathological classification of the tumors and subtypes of adenocarcinomas was achieved by two of the authors (E. T. and Y. I.) according to the 1999 WHO classification of lung tumors.⁴ Subclassification of adeno-

Figure 1. Cell types of adenocarcinomas. **A:** Hobnail cell type: apical portions of carcinoma cells protrude or bulge into the lumen. Note hobnail- or tadpole-shaped cells. **B:** Columnar/cuboidal cell type: carcinoma composed of nonciliated columnar or cuboidal cells without or with only small amounts of mucus in their cytoplasm. Apical portions of the cells are flat. **C:** Polygonal cell type: carcinoma composed of polygonal cells with or without mucus in their cytoplasm, proliferating in sheets. **D:** Goblet cell type: carcinoma cells have abundant mucus in their cytoplasm (hematoxylin and eosin staining; original magnification, $\times 200$).

carcinomas was carried out with reference to the predominant cell type occupying more than 70% of the area, except for the mixed type: 1) hobnail, 2) columnar/cuboidal, 3) mixed, 4) polygonal, and 5) goblet cell types (Figure 1).²⁰ The first consists of cells with cytoplasmic protrusions or dome formation at their apices and hobnail- or tadpole-shaped cells. The second is composed of columnar/cuboidal cells with flat apices. Cytoplasmic mucus is usually absent, and if it is present it is scanty and is located near the free cell surface. The third demonstrates a mixture of hobnail, columnar/cuboidal, and goblet cells or two of these. Most of this type consists of both the former two cells, in which each cell type occupies more than 30% of the area. Polygonal cells with or without mucus in their cytoplasm, proliferating in sheets, are sometimes observed in tumors of the first three types,

but when such areas made up more than 95% of the tumor, it was diagnosed as the polygonal cell type. The goblet cell type is composed of columnar or cuboidal cells with abundant mucus in their cytoplasm. Distribution by cell type and 1999 WHO classification of adenocarcinomas are presented in Table 1. By the WHO classification, more than half of the tumors were classified as adenocarcinomas with mixed subtypes. There were only two bronchioloalveolar carcinoma, and no papillary adenocarcinomas consisting entirely of tall columnar or cuboidal cells were observed. Almost half of the cells were hobnail type, then columnar, mixed polygonal, and goblet, in that order. Table 2 shows the relationship between WHO and the cell type classification of adenocarcinomas. More than half of the acinar and papillary adenocarcinomas, respectively, were of columnar and

Table 2. Relationship between WHO and Cell Type Classifications of Lung Adenocarcinomas

WHO Classification	No. of cases (%)				
	Cell type classification				
	Hobnail	Columnar	Mixed	Polygonal	Goblet
Acinar	0	15 (68)	2 (9)	5 (23)	0
Papillary	14 (88)	0	1 (6)	1 (6)	0
Bronchioloalveolar carcinoma	1 (50)	0	0	0	1 (50)
Solid adenocarcinoma with mucin	0	0	0	5 (100)	0
Adenocarcinoma with mixed subtypes	39 (57)	8 (12)	15 (22)	2 (3)	4 (6)

hobnail cell types. The two bronchioloalveolar carcinomas consisted primarily of hobnail and goblet cells, in one case each, and all of the solid adenocarcinomas with mucin were of the polygonal cell type. The adenocarcinomas with mixed subtypes included various cell types, of which the hobnail type was the most common.

As for the relationship between differentiation and the subtypes, many cases of papillary (94%), bronchioloalveolar (100%), and adenocarcinoma with mixed subtypes (94%) by WHO classification or hobnail (100%), mixed (89%), and goblet (100%) cell type by cell type classification were well or moderately differentiated, whereas most acinar (95%) and solid adenocarcinomas with mucin (100%) (by the WHO classification) or columnar (91%) and polygonal cell types (100%) (by the cell type classification) were moderately or poorly differentiated.

DNA Preparation, Single-Strand Conformation Polymorphism (SSCP), and DNA Sequencing

Fresh tumor samples paired with corresponding normal tissue were obtained from all patients, quickly frozen in liquid nitrogen, and stored at -80°C until DNA extraction analysis, as previously described.²¹ Genomic DNAs were prepared, and exons 4–8 and 10 of the *p53* gene were analyzed by the polymerase chain reaction (PCR)–SSCP method.²² Coding sequences including exon-intron boundaries were amplified by PCR. The sequences of primers and PCR conditions were described previously.^{23,24} The 5' end of each primer was labeled with a fluorescent marker, sense primers were labeled with 6-carboxyfluorescein, and the antisense primer was labeled with 4,7,2',7'-tetrachloro-6-carboxyfluorescein (Japan Bio Service Corp., Asaka, Japan). SSCP using ABI PRISM 377 (Perkin-Elmer Corp., Norwalk, CT) and fluorescent-labeled primers was performed at 22°C , loading onto nondenaturing 4% polyacrylamide gels with 10% glycerol. SSCP data were processed with GeneScan Analysis 2.0.2 computer software (Perkin-Elmer Corp.). When genomic DNA extracted from tumors showed a SSCP pattern different from that of corresponding normal lung tissues, both genomic DNAs were amplified with the primers in the presence of $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ to elute the shifted DNA fragment for sequence analysis. After PCR under the same cycling conditions, products were electrophoresed in nondenaturing 5% polyacrylamide gels with 10% glycerol at the most suitable temperature (exon 4, 10°C ; exons 5–7 and 10, 25°C ; exon 8, 15°C) and 35 W of constant power for 2–3 hours. The gels were subjected to drying at 80°C for 1 hour and autoradiographed at room temperature overnight. Both normal and abnormal DNA fragments were eluted from the dried gels and reamplified using the same primers and PCR conditions. To characterize *p53* gene mutations, we sequenced the reamplified DNAs using a dRhodamine terminator cycle sequencing kit (Applied Biosystems) and ABI PRISM 377. Some DNA for which mutations could not be identified by direct sequencing, despite showing abnormal bands in fluorescently labeled SSCP, were subcloned

into plasmid vector pGEM-7Zf(+) (Promega) and sequenced with an AutoRead sequencing kit (Pharmacia Biotech), using fluorescently labeled SP6, T7 primers and an A.L.F. DNA Sequencer II (Pharmacia LKB Biotechnology AB).

Statistical Analysis

To establish any correlations among the *p53* gene mutation status and clinicopathological data, the χ^2 test or Fisher's exact probability when expected values in the χ^2 test were <5 , the Mann-Whitney *U*-test and Student's *t*-test were used. Differences were considered to be significant when the *P* value was <0.05 .

Results

Frequency of p53 Mutations and Mutational Spectra

p53 Mutation

Screening of all tumor samples for *p53* mutations in exons 4–8 and 10, using a fluorescently labeled PCR–SSCP, technique revealed mutations in 68 of 151 non-small-cell lung carcinomas (45%) (Tables 1 and 3). One case (case no. 17) had two mutations: a 19-bp deletion in exon 4 and a 1-bp deletion in exon 8. Of the 68 mutations, four (6%) were located in exon 4, 17 (25%) in exon 5, 9 (13%) in exon 6, 15 (22%) in exon 7, 16 (23%) in exon 8, four (6%) in exon 10, and 4 (6%) in splicing junctions of exons. No mutations were found in normal lung tissue samples, except in patients carrying a polymorphism in exon 4, codon 72.²⁵ Histologically, a trend toward more frequent mutations in squamous cell carcinomas (58%) than in adenocarcinomas (41%) was found (Table 4 and Figure 2A), in line with earlier results of a Japanese study of mutations in exons 2–11 in 115 cases of non-small-cell lung cancer.²⁶ By the WHO classification, papillary adenocarcinomas and adenocarcinomas with mixed subtypes showed the lowest frequency of mutations, although statistically significant differences from other individual subtypes were not found (Table 1). Using our cytological classification, the highest frequency of the mutations was observed in the columnar cell type (70%), which is similar to the finding for squamous cell lesions, followed by polygonal (46%), goblet (40%), hobnail (37%), and mixed (11%) cell types. The differences compared with the latter two were statistically significant (Table 4 and Figure 2A). This was also the case for the squamous cell carcinomas (hobnail, $P = 0.048$; mixed cell, $P < 0.001$; by χ^2 test).

p53 Mutational Spectra (Table 4)

Most *p53* mutations were transitions (43%) or transversions (41%), and only 16% were deletions/insertions (Table 4). In adenocarcinomas, the frequencies of transitions and transversions were 46% and 35%, and in squamous cell carcinomas, 36% and 55%, respectively. No signifi-

Table 3. *p53* Mutations in Lung Adenocarcinomas and Squamous Cell Carcinomas

Case no.	Sex [†]	Age (years)	Smoking [‡]	Tumor						Mutation*			
				Histology [§]	Location	WHO [¶]	Cell type [#]	Diff ^{**}	pStage ^{††}	Exon (intron)	Codon	Base change ^{‡‡}	Amino acid
198	M	55	(+)	Ad	P	Mix	Hob	M	IA	4	46	Ins of 16 bp	Frameshift
138	M	44	(-)	Ad	P	Acinar	Col	P	IIIA	4	120	AAG to AGG	Lys to Arg
17	M	69	(+)	Ad	P	Mix	Col	M	IA	4	113-119	Del of 19 bp	Frameshift
										8	301	CCA to C A	Frameshift
203	F	67	(-)	Ad	P	Mix	Hob	W	IIIB	5	132	AAG to AGG	Lys to Arg
105	M	61	(+)	Ad	P	Acinar	Col	P	IIIB	5	135	TGC to TTC	Cys to Phe
11	F	72	(-)	Ad	P	Mix	Hob	W	IIIB	5	138	GCC to GTC	Ala to Val
19	F	57	(-)	Ad	P	Acinar	Col	M	IIIA	5	138	GCC to CCC	Ala to Pro
208	M	71	(+)	Ad	P	Mix	Col	M	IIIA	5	157	GTC to TTC	Val to Phe
22	M	72	(+)	Ad	P	Acinar	Col	M	IIIB	5	158	CGC to CAC	Arg to His
96	M	60	(+)	Ad	P	Solid	Poly	P	IIA	5	158	CGC to CAC	Arg to His
197	M	47	(+)	Ad	P	Solid	Poly	P	IIIA	5	158	CGC to CCC	Arg to Pro
173	M	54	(+)	Ad	P	Mix	Col	M	IA	5	158	CGC to CTC	Arg to Leu
79	M	56	(+)	Ad	P	Pap	Hob	W	IIIB	5	159	GCC to C	Frameshift
103	M	74	(+)	Ad	P	Mix	Hob	W	IA	5	175	CGC to CAC	Arg to His
134	F	26	(-)	Ad	P	Pap	Hob	W	IIIB	5	176	TGC to TTC	Cys to Phe
191	M	50	(+)	Ad	P	Acinar	Col	M	IA	5	179-185	Del of 18 bp	Frameshift
89	M	41	(+)	Ad	P	Pap	Poly	P	IB	(5)	Acceptor	ag G to at G	Splicing
160	M	50	(+)	Ad	P	Acinar	Col	M	IV	6	189	GCC to G_C	Frameshift
97	M	54	(+)	Ad	P	Acinar	Col	M	IIIA	6	198	GAA to TAA	Glu to Stop
122	F	74	(+)	Ad	P	Mix	Hob	W	IIIB	6	209	AGA to TGA	Arg to Stop
205	F	49	(-)	Ad	P	Pap	Hob	M	IB	6	213	CGA to TGA	Arg to Stop
186	M	74	(+)	Ad	P	Mix	Gob	M	IB	(6)	Donor	AG gt to AG at	Splicing
23	M	58	(+)	Ad	P	Mix	Hob	W	IA	7	234	TAC to TGC	Tyr to Cys
38	F	77	(-)	Ad	P	Mix	Hob	W	IIIB	7	237	ATG to ATT	Met to Ile
157	M	49	(+)	Ad	P	Mix	Mix	W	IIA	7	238	TGT to AGT	Cys to Ser
80	F	65	(-)	Ad	P	Mix	Gob	M	IA	7	241	TCC to TC	Frameshift
101	F	68	(+)	Ad	P	Acinar	Col	M	IIIB	7	242	TGC to TAC	Cys to Tyr
66	F	51	(-)	Ad	P	Mix	Col	W	IIIB	7	245	GGC to AGC	Gly to Ser
28	M	47	(+)	Ad	P	Acinar	Col	P	IIIA	7	245	GGC to TGC	Gly to Cys
33	F	37	(+)	Ad	P	Mix	Hob	M	IA	7	248	CGG to TGG	Arg to Trp
139	F	70	(-)	Ad	P	Mix	Hob	W	IA	7	248	CGG to CAG	Arg to Glu
3	M	73	(-)	Ad	P	Solid	Poly	P	IIIB	7	259	GAC to AAC	Asp to Ile
69	M	58	(+)	Ad	P	Acinar	Poly	P	IIIA	8	273	CGT to TGT	Asp to Cys
182	M	56	(+)	Ad	P	Mix	Hob	W	IA	8	273	CGT to TGT	Asp to Cys
49	F	49	(-)	Ad	P	Mix	Hob	W	IIIB	8	273	CGT to CAT	Asp to His
174	M	72	(+)	Ad	P	Pap	Hob	M	IA	8	273	CGT to CAT	Asp to His
100	M	48	(+)	Ad	P	Solid	Poly	P	IA	8	273	CGT to CTT	Asp to Leu
152	F	68	(-)	Ad	P	BAca	Hob	W	IA	8	273	CGT to CTT	Asp to Leu
154	M	72	(+)	Ad	P	Mix	Col	M	IV	8	274	GTT to T	Frameshift
34	M	59	(+)	Ad	P	Acinar	Col	M	IA	8	274	GTT to TTT	Val to Phe
156	M	65	(-)	Ad	P	Mix	Hob	W	IA	8	275	TGT to TAT	Cys to Tyr
155	F	63	(-)	Ad	P	Mix	Hob	W	IIIB	8	282	CGG to TGG	Arg to Trp
185	M	67	(+)	Ad	P	Mix	Hob	M	IIIB	8	305-306	Ins of 23 bp	Frameshift
15	M	64	(+)	Ad	P	Acinar	Col	M	IA	(8)	Donor	AG gt to AG tt	Splicing
83	F	65	(-)	Ad	P	Mix	Hob	W	IA	10	335	CGT to CAT	Arg to His
148	F	51	(-)	Ad	P	Mix	Mix	M	IIIA	10	341	TTC to T_C	Frameshift
200	M	71	(+)	Sq	C			P	IIIB	4	103	TAC to TAG	Tyr to Stop
146	M	76	(+)	Sq	C			M	IIIB	5	144	CAG to CCG	Gln to Pro
187	M	69	(+)	Sq	P			M	IA	5	166	TCA to TAA	Ser to Stop
70	M	59	(+)	Sq	C			M	IIIA	5	175	CGC to CAC	Arg to His
54	M	70	(+)	Sq	P			M	IIIB	5	149-175	Del of 79 bp	Frameshift
113	M	63	(+)	Sq	C			M	IIIB	6	190	CCT to C_T	Frameshift
179	M	70	(+)	Sq	P			M	IIIB	6	195	ATC to ACC	Ile to Thr
135	M	49	(+)	Sq	P			M	IIIA	6	196	CGA to CCA	Arg to Pro
7	M	71	(-)	Sq	P			M	IA	6	220	TAT to TGT	Tyr to Cys
167	M	82	(+)	Sq	P			M	IIIB	6	220	TAT to TGT	Tyr to Cys
87	M	65	(+)	Sq	P			M	IB	7	244	GGC to TGC	Gly to Cys
20	F	70	(+)	Sq	P			M	IIIB	7	245	GGC to TGC	Gly to Cys
40	M	79	(+)	Sq	P			P	IA	7	245	GGC to CGC	Gly to Arg
73	M	66	(+)	Sq	P			M	IIIB	7	245	GGC to CGC	Gly to Arg
193	M	68	(+)	Sq	C			P	IIIA	7	245	GGC to GTC	Gly to Val
188	M	53	(+)	Sq	C			M	IIIB	8	271	GAG to TAG	Glu to Stop
109	M	68	(+)	Sq	C			M	IIIA	8	273	CGT to TGT	Arg to Cys
56	M	59	(+)	Sq	C			W	IIIB	8	282	CGG to TGG	Arg to Trp
183	F	75	(-)	Sq	C			P	IIIB	8	282	CGG to TGG	Arg to Trp
159	M	51	(+)	Sq	C			M	IIA	(9)	Acceptor	agAT to tgAT	Splicing
4	M	61	(-)	Sq	P			M	IIIB	10	337	CGC to CTC	Arg to Leu
29	M	76	(+)	Sq	P			M	IB	10	342	CGA to TGA	Arg to Stop

*Ref. 24.

[†]M, male; F, female.

[‡](+), smoker; (-), nonsmoker.

[§]Ad, adenocarcinoma; Sq, squamous cell carcinoma.

^{||}Location of tumors; C, central; P, peripheral.

[¶]WHO subclassification of adenocarcinoma. Pap, papillary; BAca, bronchioloalveolar carcinoma; Solid, solid adenocarcinoma with mucin; mix, adenocarcinoma with mixed subtypes.

[#]Cell type classification; Hob, hobnail cell type; Col, columnar cell type; Mix, mixed cell type; Poly, polygonal cell type; Gob, goblet cell type.

^{**}Differentiation of the tumor. W, well differentiated; M, moderately differentiated; P, poorly differentiated.

^{††}Pathological stage.

^{‡‡}Del, deletion; Ins, insertion.

Table 4. *p53* Mutational Spectra for Types of Adenocarcinomas, and Squamous Cell Carcinomas and Relation to Smoking

Histology and smoking status	No. of cases (%)											
	Examined	With <i>p53</i> mutation	Transition				Transversion				Total	Del/Ins*
			CpG G:C to A:T	Non-CpG G:C to A:T	A:T to G:C	Total	G:C to T:A	G:C to C:G	A:T to T:A	A:T to C:G		
All cases	151	68 (45)	18 (26)	5 (7)	6 (9)	29 (43)	18 (26)	6 (9)	3 (4)	1 (2)	28 (41)	11 (16)
Histology												
Adenocarcinoma	113	46 (41)	13 (28)	5 (11)	3 (7)	21 (46)	12 (26)	2 (4)	2 (4)	0	16 (35)	9 (20)
Squamous cell carcinoma	38	22 (58)	5 (23)	0	3 (14)	8 (36)	6 (27)	4 (18)	1 (5)	1 (5)	12 (55)	2 (9)
Cell type classification of adenocarcinoma												
Hobnail cell type	54	20 (37)	9 (45)	2 (10)	2 (10)	13 (65)	3 (15)	0	1 (5)	0	4 (20)	3 (15)
Columnar cell type	23	16 (70)	2 (13)	1 (6)	1 (6)	4 (25)	7 (44)	1 (6)	0	0	8 (50)	4 (25)
Mixed cell type	18	2 (11)	0	0	0	0	0	0	1 (50)	0	1 (50)	1 (50)
Polygonal cell type	13	6 (46)	2 (33)	1 (17)	0	3 (50)	2 (33)	1 (17)	0	0	3 (50)	0
Goblet cell type	5	2 (40)	0	1 (50)	0	1 (50)	0	0	0	0	0	1 (50)
Smoking status												
Nonsmoker	55	20 (36) [†]	7 (35)	3 (15)	3 (15)	13 (65) [‡]	4 (20)	1 (5)	0	0	5 (25) [§]	2 (10)
Smoker	96	48 (50)	11 (23)	2 (4)	3 (6)	16 (33)	14 (29)	5 (10)	3 (6)	1 (2)	23 (48)	9 (19)

*Deletion/insertion.

[†]Nonsmoker vs. smoker, $P = 0.105$ (by χ^2 test).

[‡]Nonsmoker vs. smoker, $P = 0.016$ (by χ^2 test).

[§]Nonsmoker vs. smoker, $P = 0.068$ (by Fisher's exact probability test).

cant differences were observed, in agreement with a previous Japanese report.²⁶ Comparison of subtypes revealed a significant difference between hobnail and columnar cell groups: transitions were higher in the former (65%) than the latter (25%) (Figure 2B). Transversions also tended to be less frequent in the former (20%) than in the latter (50%). With the WHO subclassification, no significant differences were observed between subtypes of adenocarcinomas as to the frequencies of transitions or transversions (data not shown). We did not analyze the rates of deletions and insertions, because the proportions that represent changes

induced by endogenous *versus* exogenous mechanisms remain unclear.²⁷

Next, base substitutions were examined with reference to subtypes of adenocarcinoma and squamous cell carcinoma (Table 4 and Figure 2C). With CpG site transitions, the frequency in the hobnail cell type (45%) was higher than those for columnar cell type and squamous cell carcinomas (13% and 23%, respectively), the difference being statistically significant in the former case. On the other hand, G:C to T:A transversions tended to be rarer in hobnail cell-type lesions (15%) than in the other two types (44% and 27%, respectively). With the WHO subclassification, no such variation was noted.

Figure 2. **A:** Frequencies of *p53* mutations. **B:** Rates of transitions and transversions. **C:** Rates of G:C and A:T transitions. **D:** Smokers' and nonsmokers' rates with reference to adenocarcinoma histology and cell type. *Percentage (No. of cases/Total no. of examined cases). [‡] χ^2 test. [‡]Percentage (No. of cases/Total no. of mutated cases). [§]Fisher's exact probability test.

Strand Bias

It has been hypothesized that mutations induced by exogenous or environmental carcinogens preferentially occur in nontranscribed gene alleles.^{8,27-29} Therefore evaluating the *p53* base substitution for strand bias may also provide clues to suspected carcinogens. In our study, a marked strand bias was observed for G:C to T:A transversions: 17 of the 18 mutations were found on the nontranscribed strand, whereas the 18 G:C to A:T transitions observed in CpG sites were equally distributed on the two strands (9 vs. 9).

Smoking Status in Relation to Histology and *p53* Mutation

The percentage of smokers with squamous cell carcinomas was higher than the percentage of smokers with adenocarcinomas, and the difference was statistically significant (Figure 2D). The percentage of smokers with columnar cell lesions, 83%, was almost the same as the percentage of smokers with squamous cell carcinomas, and significantly higher than the percentages of smokers with the hobnail (44%) or mixed (39%) cell types.

Mutations were more frequent in lesions observed in smokers (50%) than in nonsmokers (36%), consistent with previous reports (Table 4).^{8,15} As for the mutational spectra, transitions were less frequent among smokers (33%) than among nonsmokers (65%), with statistical significance ($P = 0.016$, by χ^2 test). Conversely, transversions were more common among the former (48%) than the latter (25%), with a statistical trend ($P = 0.068$, by the Fisher's exact probability test). Furthermore, G:C to A:T transitions at CpG sites were preferentially found in nonsmokers (35%), and G:C to T:A transversions more frequently in smokers (29%), although the differences were not significant.

Relationship between *p53* Mutations and Clinicopathological Parameters

As clinicopathological parameters, age, gender, differentiation status, location, and stage of the tumor were adopted (Table 1). There were no significant differences in *p53* status with the first four of these. The frequency of mutations with pStages II–IV was significantly higher than that for pStage I for squamous cell carcinomas ($P = 0.038$, by Fisher's exact probability test) but not for adenocarcinomas.

Discussion

Shimosato et al classified well-differentiated adenocarcinomas into six subtypes: 1) bronchial surface epithelium type, 2) goblet cell type, 3) bronchial gland cell type, 4) nonciliated bronchiolar (Clara) cell type, 5) type II alveolar cell type, and 6) mixed cell type, based on cytological and ultrastructural features of tumor cells with consideration of histogenesis.¹⁰ We have modified his classifica-

tion and created a new cell type classification that is different in two points. The first is that adenocarcinomas are classified according to cytological features by a light microscope, and knowledge is obtained from immunohistochemical and genetic studies without any consideration of histogenesis. Metaplastic changes occur not infrequently in malignant tumors, and recently a report that biphasic tumors such as carcinosarcoma and pulmonary blastoma originate from a common progenitor cell has been published,²⁹ so that it is difficult to speculate about the cell of origin from the cell type. Therefore, we classified adenocarcinomas into five subtypes by cytological features. In our classification, the clara cell type and type II cell type were combined as the hobnail cell type, because these types have the same cytological features and immunohistochemically they are usually found to be mixed.^{10,30,31} The columnar/cuboidal type included the bronchial surface epithelium and bronchial gland cell types, with no or scanty mucus in their cytoplasm, the cell apices being flat. The goblet cell type was composed not only of goblet cells but also of bronchial gland cells with abundant mucus in their cytoplasm. This type is reported to have an especially high *K-ras* mutation rate.²⁰ The second point is that the polygonal cell type was categorized first by us, because such cells were found in poorly differentiated adenocarcinomas but not in well-differentiated ones, which was the object of Shimosato's classification.

As for distribution of the subtypes, the hobnail cell type was the most common (48%), almost the same as the figure (39%) for the clara cell type + type II cell in Shimosato's classification.¹⁰ The columnar/cuboidal cell type was the second most frequent (20%), again in the same range as reported earlier (22–34%) for the bronchial surface epithelium type.^{10,32} The frequency of the mixed type (16%) and those of the other cell types (less than 12%) were also similar for the two classifications.^{10,32} Therefore, our cases examined can be considered to be representative for surgically resected adenocarcinomas of Japan in terms of subtype distribution.

When the predominant cell type of a tumor occupies around 70% of the tumor area, it is sometimes difficult to classify the cell type. Examination for reproducibility resulted in 17 of 113 cases being classified as other cell types, the concordance rate being 85%. The 17 cases were seven hobnail cell, four mixed, three polygonal, two cuboidal, and one goblet type in the original classification. All of the hobnail cases were changed to mixed, and two of four mixed ones to hobnail. Thus the differentiation between hobnail and mixed cell types was imperfect. However, when the relationship between *p53* mutational spectra and the newly classified cell types was examined, the results were the same as originally found.

Exons 4–8 and 10 of the *p53* gene were examined for mutations in this study because they encompass more than 98% of the mutations reported in carcinomas so far.²⁶ Epidemiologically, the presence of *p53* mutations in lung cancers is closely associated with lifetime tobacco consumption, typically with G:C to T:A transversions and a predominance of guanine residues on the nontranscribed DNA strand.^{8,12,15,33,34} When lung cancers are

classified histologically, an altered *p53* mutation status with G:C to T:A transversion is marked in squamous cell carcinomas, which are strongly associated with tobacco smoke, whereas this is less clear for adenocarcinomas, which are only weakly linked with the smoking habit.⁶⁻⁸ Experimentally, compounds included in cigarette smoke, such as benzo[a]pyrene, are reported to produce G:C to T:A transversions.¹⁷ Although the same changes can also be induced by endogenous agents like oxygen radicals, a nontranscribed bias has not been reported in such cases.^{35,36}

In our study, when adenocarcinomas were subclassified by cell type, the columnar cell lesion showed high mutation and transversion rates with a nontranscribed bias, and a strong association with smoking, like that for squamous cell carcinomas, was apparent. On the other hand, hobnail cell-type adenocarcinomas showed a significantly weaker association with tobacco smoke, so that other causative factors must be considered. The finding of a high rate of G:C to A:T transitions at CpG sites with no strand bias is interesting in this context. This type of mutation is suspected to be caused mainly by deamination of 5-methylcytosine at CpG sites and occurs spontaneously without exogenous mutagens.³⁷⁻³⁹ Whether other agents might also play a role remains unclear, but, in at least a subset of hobnail cell-type adenocarcinomas, a contribution of G:C to A:T transitions at CpG sites may be hypothesized. Further studies are now needed to test this.

Hypermethylation of the promoter region of the *p16* tumor suppressor and estrogen receptor genes and loss of heterozygosity and exon deletions within the fragile histidine triad (FHIT) gene have been reported to be associated with the smoking habit in lung cancer patients.⁴⁰ Relationships between these genetic alterations and cell types should be examined to confirm our results.

In conclusion, the present study for the first time clearly showed that lung adenocarcinomas could be subclassified in terms of etiology in addition to morphology. There appear to be two major subtypes, one probably caused by tobacco smoke and the other mainly associated with endogenous, possibly spontaneous mutations. Cell type classification is thus useful for a distinction of differences that extend beyond the morphology level.

For prevention of lung adenocarcinomas, elucidation of what endogenous mechanisms are actually involved is an important next step, in addition to stopping tobacco smoking. In the future, to achieve better clinical control, it will also be necessary to investigate tumor type-specific differences in clinical characteristics, prognosis, and response to chemotherapy, radiotherapy, or innovative therapies.

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