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Evaluation of p53 Gene Mutation and Loss of Heterozygosity of 3p, 9p and 17p in Precancerous Lesions of 29 Lung Cancer Patients

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ABSTRACT

To assess the multiple steps involved in carcinogenesis we evaluated p53 gene mutation frequencies, and loss of heterozygosity (LOH) of chromosomes 3p, 9p and 17p in hyperplasia, squamous metaplasia, dysplasia, squamous cell carcinoma in-situ and early squamous cell carcinoma of the bronchial epithelium, using immunohistochemical, polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) and microsatellite analysis. Overexpression of p53 oncoprotein was observed in 31% of mild dysplasia, 50% of moderate and 67% of severe dysplasia. Mutation of the p53 gene, however, was confirmed only in 2 severe dysplasias. LOH of 3p, 9p and 17p were detected in 8% of mild and 6% of moderate dysplasias. Among other types of bronchial lesions, two columnar cell hyperplasias showed LOH of 3p and 17p, respectively. These results suggest that LOH of 3p or 9p may be a rare but early event in the development of squamous cell carcinoma of the lung. In particular, deletion of 3p21.3 is a relatively common event in premalignancy. The results also suggest that p53 gene abnormalities occur during the following stage of carcinogenesis.

Key words: *Bronchial dysplasia, Microdissection, p53 gene mutation, Loss of heterozygosity*

The histogenesis of central-type lung cancer, in particular squamous cell carcinoma, is not fully understood. However, it has been proposed that dysplasia of the bronchial epithelium (defined as atypical squamous cell proliferation in the bronchial epithelium) is a precancerous lesion, based on histological examination of non-cancerous bronchial epithelium in lung cancer patients, especially those in high risk groups[8,9,21,23,28,36]. In these patients, dysplasia was detected more frequently than in the controls.

Recently, the concept of multistep carcinogenesis has been confirmed in certain cancers, including those of the colon and liver. This involves the accumulation of changes in genes such as APC, K-ras and p53 at each step. However, knowledge regarding such changes in lung cancer is limited. So far, mutation of p53 and K-ras genes and deletion of the short arm at chromosome 3 (3p) have been identified in lung carcinoma[11,22,31]. Loss of heterozygosity (LOH) of 3p or 17p has also frequently been observed in all histological types of lung carcinoma[32,38], and LOH of 9p has been noted in non-small cell lung cancer, in particular squamous cell carcinoma[20,35]. However, precisely which of these are essential genetic changes at the precancerous or early cancerous stages of squamous cell carcinoma remains unclear[10]. Thus, it is important to investe the genetic changes occurring in dysplasia and other hyperplastic lesions of the bronchial epithelium, and comparison with those evident in in-situ or early stage carcinoma. Recent progress in techniques such as microdissection from formalin-fixed, paraffin-embedded tissue and LOH analysis using polymerase chain reaction (PCR) methods have enabled the determination of genetic changes in small lesions that can only be identified histologically. In the present study we examined mutation of the p53 gene by immunohistochemical, polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) and direct sequencing analyses, as well as LOH of 3p, 9p and 17p, in various types of bronchial lesions in order to ascertain the role of these genetic changes in the histogenesis of squamous cell carcinoma of the central bronchus.

MATERIAL AND METHODS

Tissue preparation

Dysplastic lesions were obtained from surgically

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resected lung tissue in 29 patients with lung cancer. One of these patients had small cell carcinoma and the remaining 28 had squamous cell carcinoma. Dysplasias and other lesions were detected by histological examination of serial sections cut at 2–4 mm intervals from formalin-fixed, paraffin-embedded tissue (12 to 52 specimens for each case).

Eleven 6μm-thick serial sections including the dysplasia and any other lesions were cut from each block and the last section was stained with hematoxylin and eosin to confirm the presence of lesions. None of the lesions contained carcinoma cells. The remaining sections were used for immunohistochemical examination. The lesions included 16 mild dysplasias, 12 moderate and 9 severe dysplasias. Five squamous metaplasias, 6 columnar cell hyperplasias, 4 squamous cell carcinomas in-situ and 5 early stage squamous cell carcinomas, which had no invasion over the bronchial cartilage, were also studied. A further 15 lesions of normal bronchial epithelium were examined as controls. The histological criteria used were those described by Auerbach[1,2] and the World Health Organization (WHO): dysplasia was defined as squamous metaplasia associated with nuclear pleomorphism, an irregular nuclear arrangement and increased N/C (nucleus/cytoplasm) ratio. Dysplasia was divided into three categories: mild, moderate and severe. In mild dysplasia, dysplastic cell proliferation was limited to one-third of the epithelial layer. In moderate dysplasia, dysplastic cells occupied two-thirds of the epithelial layer. In severe dysplasia, dysplastic cells replaced the entire epithelial layer, but flattened squamous cells were noted in the superficial zone. Squamous cell carcinoma in-situ was defined as a lesion in which dysplastic cells replace the entire epithelial layer with a disordered cellular arrangement, but no invasive growth into the subepithelium.

Immunohistochemistry

Immunohistochemical staining for p53 protein was performed by the streptavidin-biotin complex (SAB, Nichirei, Japan) method. The antibody used was DO-7 (Novocastra Laboratory, England), a monoclonal antibody against both wild and mutant types of human p53 protein. Before staining, microwave antigen retrieval was performed by microwaving the sections in 10 mM citrate buffer (pH6.0) for 15 min, using a 500 W microwave oven at full power.

DNA Extraction

The sections were deparaffinized in xylene, which was then removed using a graded alcohol series. After rehydration in sterile deionized water, the lesions were selectively dissected from unstained tissue sections using a stereoscopic microscope. The dissected tissues were placed in microfuge tubes, dried and incubated with 100μl buffer containing 50 mM Tris (pH8.3), 1 mM EDTA, 0.5% Tween 20 and 800μg/ml proteinase K[37] overnight at 37°C. The proteinase was then inactivated at 95°C for 8 min. Insoluble material was pelleted by centifugation and DNA was purified from aliquots of the supernatant by the usual method[25].

Table 1. Dinucleotides Repeat Markers

Marker	Chromosome location	Sequences of primers (5'–3')	Annealing temperature (°C)
D3S643	3p21.3	GACAGAACTGCCAAACCATCCCAC TATGTGCTCCAGGCTGGGTAACAG	55
D3S663	3p21.3	CCTGGCTCTGTGAGGGACA CCTGCATGGGCTTGTCTAGTC	60
D3S1007	3p21.3–22	GGAGGGTCACTTGAGTCTAGGAG ATTTGCCACCATGCCTGGCTAG	58
D3S1110	3p25	TCGACATCAGAATGCCCTATAC GGTGTCTGCCACTGTTCTGGG	55
D3S1228	3p14.1–14.3	TCCTTAACTCTTTCTCTGTGAGTTG TCTAGGAAAGGGATTAGGAAGGA	58
INFA	9p21	TGCGCGTTAAGTTAATTGGTT CTAAGGTGGAAACCCCCACT	55
D9S144	9ptel-22	GGATAAATACACTGGAAAAGAGAT AAATATTATAGCAAGTTAATTACTGAA	55
D9S171	9pcen-21	ACCCTAGCACTGATGGTATAGTCT AGCTAAGTGAACCTCATCTCTGTCT	55
TP53	17p13	AGGGATACTATTCAGCCCGAGGTG ACTGCCACTCCTTGCCCCATTC	58

Table 2. Overexpression of Oncoprotein and Mutation of p53 Gene, and Loss of heterozygosity of 3p, 9p and 17p in Various Bronchial Lesions

Bronchial lesions	No. of lesions	No. of lesions with positive p53 overexpression (%)	No. of lesions with mutation of p53 gene (%)	No. of lesions with LOH (%)		
				3p	9p	17p
Normal	15	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Squamous metaplasia	5	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Columnar cell hyperplasia	6	1 (17)	0 (0)	1 (17)	0 (0)	1 (17)
Dysplasia						
Mild	16	5 (31)	0 (0)	0 (0)	1 (6)	0 (0)
Moderate	12	6 (50)	0 (0)	1 (8)	0 (0)	0 (0)
Severe	9	6 (67)	2 (22)*	1 (11)	1 (11)	2 (22)
Squamous cell carcinoma in-situ	4	2 (50)	1 (25)[2*]	2 (50)	1 (25)	0 (0)
Early stage squamous cell carcinoma	5	3 (60)	1 (20)[3*]	1 (20)	1 (20)	1 (20)

* case 4, exon 6/codon 196 (CGA→CCA), case 14, exon 8/codon 268 (AAC→AGC)
[2*] case 6, exon 8/codon 270 (TTT→TGT)
[3*] case 17, exon 8/codon 298 (GAG→GTG)

Detection of p53 mutation and sequencing

PCR-SSCP analysis was performed to detect p53 mutations in the region between exon 5 and 8. The oligonucleotide primers and PCR conditions used to amplify genomic DNAs were identical to those previously described[14]. A 98μl aliquot of stop solution was added to 2μl of PCR product, the mixture was denatured heatedly, and the product were separated by electrophoresis through a 6% polyacrylamide gel with Tris-borate-EDTA buffer. Exon 5, 7 and 8 were electrophoresed at room temperature, and exon 6 was electrophoresis in a cold room (4°C) with a cooling fan. The gel was dried and exposed to X-ray film with an intensifying screen at room temperature. Any genomic DNA in dysplasias that showed an altered mobility shift in this PCR-SSCP analysis was reamplified in a separate PCR and subjected to direct sequencing with a cloning step, using a slight modification of a standard protocol as previously described[14,30]. Sequences were also obtained using a Sequenase PCR product sequencing kit (United States Biochemicals, USA).

PCR Amplification and Analysis on LOH Using Microsatellite Maker

The primers used in this study are shown in Table 1, and was identical to that used in previous studies[4,17,18]. PCR was performed in 10μl reaction volumes, containing 5 pmol of each primer, standard PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$), 200μM of each of dATP, dGTP, dTTP and dCTP, 0.5μCi [α-^{32}P] dCTP, 1μl of DNA solution and 0.2 units Taq DNA polymerase. The reaction mixtures were heated to 94°C for 5 min, then subjected to 40 PCR cycles comprising denaturation for 1 min at 94°C, primer annealing for 2 min at the appropriate annealing temperature (55–60°C), strand elongation for 1.5 min at 72°C and final elongation for 10 min at 72°C. After PCR amplification, 2μl of the reaction mixture was denatured with 95% formamide and electrophoresed through 7M urea 6% polyacrylamide gels for 1–1.5 hour at 60 W, followed by autoradiography. A negative control was incorporated into each PCR run. Abnormal samples were repeatedly examined by independent PCRs and separate gel loading in order to ensure reproducibility.

Determination of allelic loss

Normal DNA samples polymorphic at a given locus were considered informative. The signal intensity of these fragments was determined by visual examination and/or densitometry. LOH was considered to have occurred when one of the two alleles showed less than 50% of the intensity shown by the corresponding normal tissue DNA.

RESULTS

Immunohistochemistry of p53 protein

Lesions were considered positive for p53 when more than 10% of cells were positive for DO-7 antibody. The proportion of positive lesions was 31% in mild, 50% in moderate and 67% in severe dysplasia (Table 2). In mild dysplasia, the positive

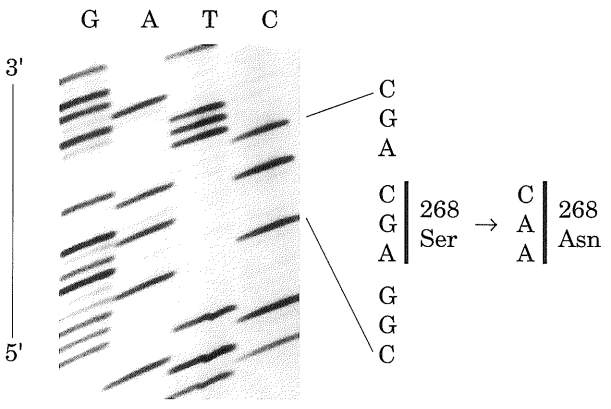

Fig. 1. DNA sequence analysis of p53 gene, exon 8 in severe dysplasia (case 14).

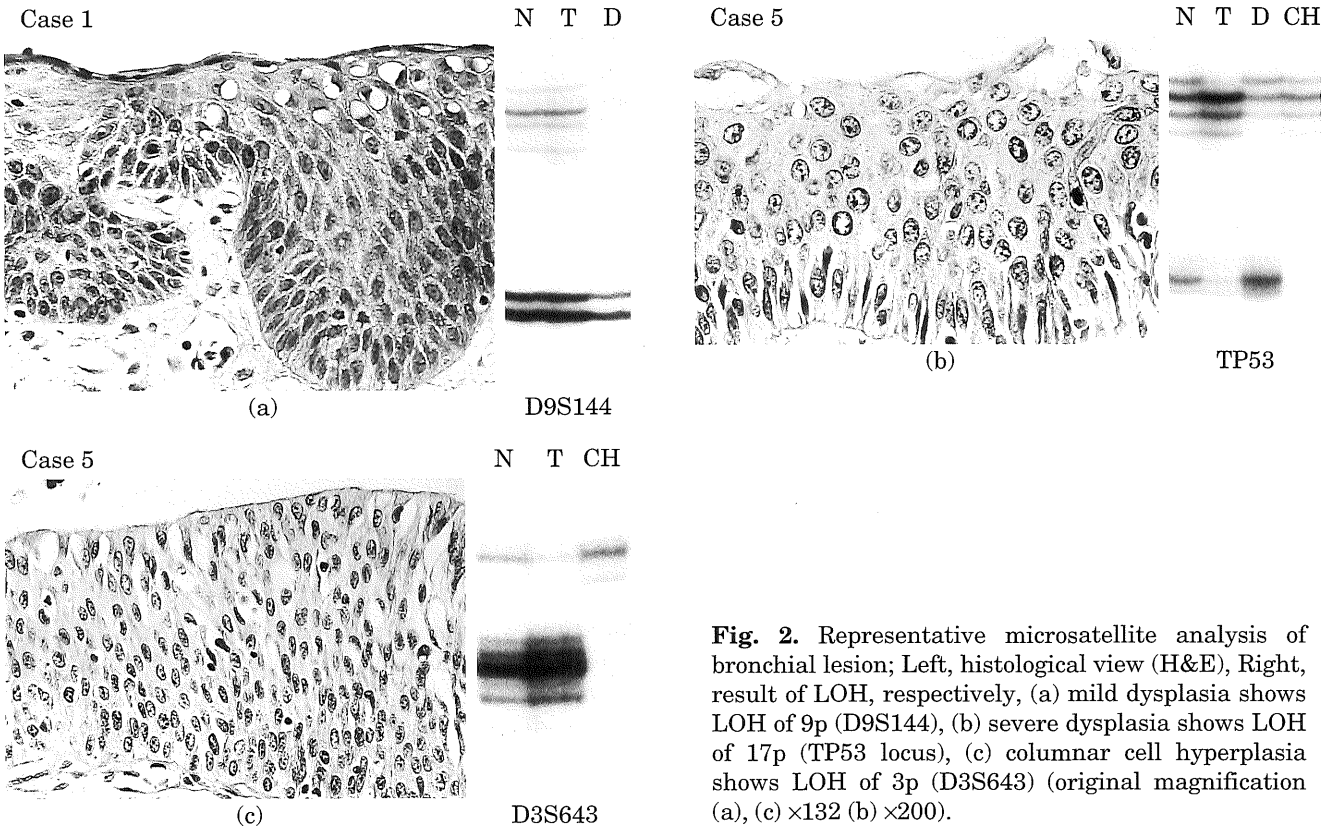

Fig. 2. Representative microsatellite analysis of bronchial lesion; Left, histological view (H&E), Right, result of LOH, respectively, (a) mild dysplasia shows LOH of 9p (D9S144), (b) severe dysplasia shows LOH of 17p (TP53 locus), (c) columnar cell hyperplasia shows LOH of 3p (D3S643) (original magnification (a), (c) ×132 (b) ×200).

cells were noted mainly in the basal and suprabasal zones, while positive cells were noted throughout the entire epithelial layer in moderate and severe dysplasia. Interestingly, one columnar cell hyperplasia adjacent to a severe dysplasia was also positive.

Mutation of the p53 gene

The results of the PCR-SSCP analysis are shown in Table 2. Sequencing analysis showed the following missense mutations in 2 dysplasias: codon 196, CGA→CCA (Arg→Pro), in case 4 and codon 268, AAC→AGC (Asn→Ser), in case 14 (Fig. 1). Strong overexpression of p53 protein was detected in these two lesions. No mutation was detected in the mild or moderate dysplasias. Among 4 squamous cell carcinomas in-situ, a mutation in codon 270 of the p53 gene without p53 protein overexpression, TTT→TGT (Phe→Cys), was found in case 6. Among the 5 patients with early stage carcinoma, a missense mutation in codon 298, GAG→GTG (Gul→Val) was detected in one with p53 protein overexpression.

Table 3. Loss of Heterozygosity and p53 Gene Mutation in Bronchial Lesions

		Microsatellite markers										p53 mutation	
		3p					9p			17p			
Case	Bronchial lesions	D3S643	D3S663	D3S1007	D3S1100	D3S1228	INFA	D9S144	D9S171	TP53	p53 over-expression	Exon/ codon	Base/ amino acid change
1	Dys, mild	he	he	he	NI	NI	he	LOH	NI	he	+		
	Dys, moderate	LOH	he	he	NI	NI	he	he	NI	he	+		
2	CIS	he	LOH	LOH	he	he	NI	he	LOH	NI	–		
4	Dys, severe	he	he	he	he	he	he	he	he	he	+	exon 6/196	CGA→CCA/Arg→Pro
5	Dys, severe	LOH	LOH	NI	he	he	LOH	he	he	LOH	+		
	CH	LOH	he	NI	he	he	he	he	he	he	–		
	CH	he	he	NI	he	he	he	he	he	LOH	+		
6	CIS	he	he	LOH	NI	NI	he	NI	NI	he	–	exon 8/270	TTT→TGT/Phe→Cys
7	SCC, early	he	NI	he	he	NI	LOH	he	he	he	–		
14	Dys, severe	NI	NI	he	NI	he	NI	NI	NI	LOH	+	exon 8/268	AAC→AGC/Asn→Ser
17	SCC, early	he	he	he	he	he	NI	NI	he	LOH	+	exon 8/298	GAG→GTG/Glu→Val
22	SCC, early	NI	NI	LOH	he	he	ho	he	he	NI	–		

LOH: loss of heterozygosity he: heterozygous NI: not informative (homozygous) Dys: dysplasia
CH: columnar cell hyperplasia CIS: squamous cell carcinoma in-situ SCC: squamous cell carcinoma

Analysis on LOH

The results of the LOH analysis are shown in Table 2. LOH of 3p was noted in one moderate and one severe dysplasia, while LOH of 9p was observed in one mild (Fig. 2a) and one severe dysplasia. LOH of 17p was found in two severe dysplasias (Fig. 2b). Dysplasias were observed in cases 1 and 5, both of which had multiple dysplastic and hyperplastic lesions. Case 17 had multiple squamous cell carcinomas without dysplasia, and showed LOH of 17p and p53 gene point mutation without deletion of 3p. All of the dysplastic lesions with LOH of 17p showed overexpression of p53 protein. The proportion of lesions displaying LOH tended to increase with the degree of dysplasia. Among other types of lesions, two columnar cell hyperplasias showed LOH of 3p and 17p (Fig. 2c). None of the squamous metaplastic lesions showed LOH. In squamous cell carcinoma in-situ, one showed LOH of 3p and 9p, and one showed LOH of 3p. Of three early stage squamous cell carcinomas, one showed LOH of 3p, one of 9p and one of 17p, and one showed p53 point mutation with deletion of the p53 gene. Table 3 summarizes the lesions with LOH and mutation.

DISCUSSION

Several studies have immunohistochemically analyzed p53 protein in bronchial lesions. Bennett et al showed that p53 protein accumulated infrequently in normal or metaplastic mucosa, while dysplasias showed an increasing tendency to accumulate p53 protein as they became more severe. In that study, accumulation of p53 protein was observed in 29.5% of mild dysplasias, 26.9% of moderate and 59.7% of severe dysplasias and in 79.5% of invasive carcinoma[3]. Another study demonstrated the presence of p53 protein in 14% of mild, 25% of moderate and 59% of severe dysplasias[34]. Direct sequence analysis of p53 and examination of LOH of 17p had also confirmed p53 inactivation in many neoplasms[12]. Further molecular analyses in preinvasive lesions have suggested that both deletion of 17p and p53 mutations might take place during the early stages of carcinogenesis in the lung[27,28].

Mutations are scattered throughout the p53 coding region, and although no particular hotspot has been identified, 70% mutations are found in exons 5–8 in lung cancer[31]. In the previous study[30], our SSCP analysis revealed that 95% of p53 mutaions occured in exons 5–8. In the present study, immunohistochemical staining of p53 protein increased with the degree of dysplasia, in agreement with previous reports, although p53 mutations were not detected by SSCP. We detected p53 mutations in dysplasias using microdissected formalin-fixed, paraffin-embedded tissue obtained from serial bronchial sections. Almost all the dysplastic lesions examined in the previous studies were obtained from frozen surgical sections or from biopsy specimens taken from tissue adjacent to the tumor[27,28]. The present study also revealed the presence of p53 mutations in two severe dysplasias. One of these also showed LOH of 17p, and both strongly overexpressed p53 protein. These results indicated that p53 abnormalities in severe dysplasia are similar to those arising in squamous cell carcinoma in-situ or early stage squamous cell carcinoma. Using a plaque assay technique, Chung et al demonstrated that adjacent, physically distinct bronchial abnormalities are clonally related. The same p53 mutation detected in a tumor was present in a small proportion of cells in an adjacent squamous metaplasia[6].

Only a few studies have analyzed genetic changes in 3p, 9p and 17p in bronchial lesions. Sunderesan et al analyzed genetic changes using PCR-RFLP in dysplastic lesions adjacent to invasive carcinoma. They demonstrated LOH of 3p in 3 of 6 dysplastic lesions using the markers D3S2 (3p21-cent) and D3F15S2 (3p21-tel) and LOH of 17p in 1 of 6 others using an RFLP marker for the AccII site[29]. Chung et al performed a detailed investigation of the genetic changes of preinvasive lesions adjacent to invasive squamous cell carcinoma. They showed that allelic loss of chromosome 3p occurred prior to LOH of the p53 gene and that deletions of chromosome 3p progressed sequentially[13]. These investigations revealed important knowledge of the genetic changes bearing on the progression of squamous cell carcinoma. In another study, LOH of 3p was detected in 76% of hyperplasias, 86% of dysplasias and in 100% of noninvasive carcinomas[15]. Identical specific alleles were also lost in 78% of preneoplastic lesions and in the corresponding carcinomas. LOH of 9p was also detected in 38% of hyperplasias, 80% of dysplasias and in 100% of CIS; these changes occurred during the earliest stage of carcinogenesis in the lung[20]. In their studies, analysis of genetic changes in cases that contained multiple preneoplastic lesions showed the higher frequencies of LOH of 3p, 9p and 17p, while a study of Sato et al revealed that frequencies of LOH were 81%, 55% and 88% in squamous cell carcinoma of lung, respectively[26].

In the present study, the frequencies of LOH of 3p and 9p were lower than those in the previous reports. Two reasons may account for this. Firstly, in some previous reports, precancerous lesions such as dysplasia (related to squamous cell carcinoma) and atypical adenomatous hyperplasia (related to adenocarcinoma) were evaluated without distinguishing the characteristics of each of the genetic changes. Secondly, in our study, dysplasias separate from the tumor were identified using a bronchial step-cut method and among the 29 cases that contained multiple dysplastic lesions were mixed cases containing sporadic dysplasia.

Unlike previous studies, we did not analyze primary cases as those which multiple dysplastic lesions. LOH of 3p and the p53 gene were detected 25% and 50% respectively in severe dysplasia. These frequencies in multiple dysplastic lesions are similar to those obtained in previous studies, and were higher than when cases of multiple dysplastic lesions were included with the other cases.

In the present study, LOH of 3p in dysplasias, including squamous cell carcinomas in-situ and early stage squamous cell carcinomas, was confined to 3p21.3, which is identical to the results of Chung et al[5]. 3p21.3 has previously been identified as one of the regions which may contain a tumor-suppressor gene for renal cell carcinoma, ovarian cancer and lung cancer[7,11,19]. Thus, in the progression of carcinogenesis, deletion of 3p21.3 may be of special significance in tumor progression from bronchial pre-invasive lesions to squamous cell carcinoma. The difference between the lesions with and without allelic loss of 3p correlated with whether the lesion is reversible to normal bronchial epithelium. We found that the 3p locus was deleted in more mildly dysplastic lesions adjacent to invasive lesions, suggesting that lesions with allelic loss of 3p proceed to more malignant potential lesions, to squamous cell carcinoma, and that morphologically mild dysplastic lesions with LOH of 3p finally progress to invasive tumors. In our study, mild and moderate dysplasia showed LOH of 9p and 3p, respectively, in cases that contained multiple dysplastic lesions. The identical frequency of 3p and 9p losses in dysplastic samples indicates that these two alterations are early events in bronchial carcinogenesis, particularly, in cases that contained multiple preneoplastic lesions. These results suggest that LOH of 3p occurs earlier in carcinogenesis than LOH of 17p or p53 gene mutation.

In addition to dysplasia, we also studied the genetic changes in hyperplastic and metaplastic lesions, such as columnar cell hyperplasia and basal cell. Interestingly, two columnar cell hyperplasias showed LOH of 3p and 17p. Thiberville L. et al observed LOH of 5q21 in reactive columnar cell hyperplasia/metaplasia in addition to genetic abnormalities of 3p or 9p[33]. We observed allelic loss of 3p in basal cell hyperplasia in the other part of the present study. Further studies of columnar cell hyperplasia are therefore required to determine whether it is also a precancerous lesion of the bronchus. In addition, evaluation is necessary, in hyperplastic lesions, of how the degree of abnormality of 3p and the p53 gene progresses to malignant lesions.

Recently, two closely related members of the cyclin-dependent kinase inhibitor family, named $p16^{INK4}$/MTS1/CDKN2 and $p15^{INK4B}$/MTS2, were isolated from the chromosomal region 9p21. Also, the p16 gene was reported to be a good candidate for a tumor suppressor gene involved in the oncogenesis of familial and sporadic melanomas[16]. A high frequency of homozygous deletion of the p16 gene has also been reported in various cultured tumor cell lines[24]. Washimi et al showed that p16 and/or p15 alterations occurred in 6 of 20 (30%) non-small cell lung cancer cell lines compared with 0 of 20 (0%) small cell lung cancer cell lines[35]. Our results also show that alteration of chromosome 9p occurs in squamous cell carcinoma in-situ, early stage squamous cell carcinoma and severe dysplasia. The mild dysplasia in case 1 was shown to LOH of chromosomal region 9p21. Thus, our observation supports the hypothesis that LOH of 9p is also an early genetic change in the development of squamous cell carcinoma.

Based on the above considerations, p53 mutation and LOH of 17p are believed to have equal significance in the histogenesis of the early stage of squamous cell carcinoma. Although we examined the regions between exon 5 and 8 of the p53 gene, it is possible that other exons also contain mutations. LOH of one allele without mutation in the other allele has been observed in small-sized small cell carcinoma. The present study suggests that loss of only one allele without p53 gene mutation in its counterpart carries the potential for tumorigenesis.

In conclusion, we have described a number of genetic abnormalities in bronchial lesions, including various degrees of dysplasia and columnar cell hyperplasia, that may possess the biological potential to progress to cancerous lesions. However, the sequence of occurrence of genetic abnormalities remains unclear and future research will focus on this area.

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REFERENCES

1. **Auerbach, O., Petrick, T.G., Stout, A.P., Statsinger, A.L., Muehsam, G.E., Forman, J.B. and Gere, J.B.** 1956. The anatomical approach to the study of smoking and bronchogenic carcinoma. A preliminary report of forty-one cases. Cancer **9:** 76–83.
2. **Auerbach, O., Gere, J.B., Forman, J.B., Petrick, T.G., Smolin, H.J., Muehsam, G.E., Kassouny, D.Y. and Stout, A.P.** 1957. Changes in the bronchial epithelium in relation to smoking and cancer of the lung. N. Engl. J. Med. **256:** 97–104.
3. **Bennett, W.P., Colby, T.V., Travis, W.D., Borkowski, A., Jones, R.T., Metcalf, R.A., Samet, J.M., Takeshima, Y., Vahakangas, K.H.,**

Soini, Y., Paakko, P., Welsh, J.A., Trump, B.F. and Harris, C.C. 1993. p53 protein accumulates frequency in early bronchial neoplasia. Cancer Res. **53:** 4817–4822.

4. **Cannon-Albright, L.A., Goldner, D.E., Meyer, L.J., Lewis, C.M., Anderson, D.E., Foutain, J.W., Hegi, M.E., Wiseman, R.W., Petty, E.M., Bale, A.E., Olopade, O.I., Diaz, M.O., Kwiatkiwski, D.J., Piepkon, M.W., Zone, J.J. and Skolnic, M.H.** 1992. Assignment of a locus for familial melanoma, MLM, to chromosome 9p13-9p22. Science **258:** 1148–1152.
5. **Chung, G.Y.T., Sundaresan, V., Hasleton, P., Rudd, R., Taylor, R. and Rabbits, P.H.** 1995. Sequential molecular genetic changes in lung cancer development. Oncogene **11:** 2591–2598.
6. **Chung, G.T.Y., Sundaresan, V., Hasleton, P., Rudd, R., Taylor, R. and Rabbits, P.H.** 1996. Clonal evaluation of lung tumors. Cancer Res. **56:** 1609–1614.
7. **El-Nagger, A.K., Batsakis, J.G., Wang, G. and Lee, M.S.** 1993. PCR-based RFLP screening of the commonly delated 3p loci in renal cortical neoplasms. Diagn. Mol. Pathol. **2:** 269–276.
8. **El-Naggar, A.K., Hurr, K., Barsakis, J.G., Luna, M.A., Goepfert, H. and Huff, V.** 1995. Sequential loss of heterozygosity at microsatellite motifs in preinvasive and invasive head and neck squamous cell carcinoma. Cancer Res. **55:** 2656–2659.
9. **Gao, H., Wangw, L.D., Zhous, Q., Hong, J.Y., Huang, T.Y. and Yung, C.S.** 1994. p53 tumor suppressor gene mutation in early esophageal precancerous lesions and carcinoma among high-risk population in Henan, China. Cancer Res. **54:** 4342–4346.
10. **Gazdar, A.** 1994. The molecular and cellular basis of human lung cancer. Anticancer Res. **13:** 2095–2097.
11. **Hibi, K., Takahashi, T., Yamakawa, K., Ueda, R., Sekido, Y., Ariyoshi, Y., Suyama, M., Takagi, H., Nakamura, Y. and Takahashi, T.** 1992. Three distinct regions involved in 3p deletion in human lung cancer. Oncogene **7:** 445–449.
12. **Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C.C.** 1991. p53 mutations in human cancers. Science **253:** 49–53.
13. **Horio, Y., Takahashi, T., Kuroishi, T., Hibi, K., Suyama, M., Niimi, T., Shimokata, K., Yamakawa, K., Nakamura, Y., Ueda, R. and Takahashi, T.** 1993. Prognostic significance of p53 mutation and 3p deletion in primary resected non-small cell cancer. Cancer Res. **53:** 1–4.
14. **Horio, Y., Suzuki, H., Ueda, R., Koshikawa, T., Sugiura, T., Ariyoshi, Y., Takahashi, T. and Takahashi, T.** 1994. Predominantly tumor-limited expression of a mutant allele in a Japanese family carrying a germline p53 mutation. Oncogene **9:** 1231–1235.
15. **Hung, J., Kishimoto, Y., Sugio, K., Virmani, A., Mclntire, D.D., Minna, J.D. and Gazdar, A.F.** 1995. Allele-specific chromosome 3p deletions occur at an early stage in the pathogenesis of lung carcinoma. JAMA **273:** 558–563.
16. **Hussussian, C.J., Struewing, J.P., Goldstein, A.M., Higgins, P.A.T., Ally, D.S., Sheahan, M.D., Clark, Jr. W.H., Tucker, M.A. and Dracopoli, N.** 1994. Germline p16 mutations in familial melanoma. Nat. Genet. **8:** 15–21.
17. **Jones, M.H. and Nakamura, Y.** 1992. Detection of loss of heterozygosity at the human TP53 locus using a dinucleotide repeat polymorphism. Genes Chrom. **5:** 89–90.
18. **Jones, M.H., Yamakawa, K. and Nakamura, Y.** 1992. Isolation and characterization of 19 dinucleotides repeat polymorphism on chromosome 3p. Hum. Mol. Genet. **1:** 131–133.
19. **Jones, M.H. and Nakamura, Y.** 1992. Deletion mapping of chromosome 3p in female genital tract malignancies using microsatellite polymorphism. Oncogene **7:** 1631–1634.
20. **Kishimoto, Y., Sugio, K., Hung, J.Y., Virami, A.K., Mcintire, D.D., Minna, J.D. and Gazdar, A.F.** 1995. Allele-specific loss in chromosome 9p loci in preneoplastic lesions accompanying non-small-cell lung cancers. J. Natl. Cancer Inst. **87:** 1224–1229.
21. **Klein, K., Vignaud, M., Sadmi, M., Plenat, F., Borelly, J., Duprez, A., Martinet, Y. and Martinet, N.** 1993. Squamous metaplasia expression of proto-oncogene and p53 in lung cancer patients. Lab. Invest. **68:** 26–32.
22. **Mitsudomi, T., Steinberg, S.M., Oie, J.L., Muishine, R., Pheips, J.V., Minna, J.D. and Gazdar, A.F.** 1991. Ras gene mutations in non-small cell lung cancers are associated with shortened survival irrespective of treatment intent. Cancer Res. **51:** 4999–5010.
23. **Nees, M., Homann, N., Discher, H., Andl, T., Enders, C., Herold-Mende, C., Schuhmann, A. and Bosch, FX.** 1993. Expression of mutated p53 occurs in tumor-distant epithelia of head and neck cancer patients: a possible molecular basis for the development of multiple tumor. Cancer Res. **53:** 4189–4196.
24. **Nobori, T., Miura, K., Wu, D.J., Lois, A., Takabayashi, K. and Carson, D.A.** 1994. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature **368:** 753–756.
25. **Sambrook, T., Fritsch, E.F. and Maniatis, T.** 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press: New York.
26. **Sato, S., Nakamura, Y. and Tsuchiya, E.** 1994. Difference of allelotype between squamous cell carcinoma and adenocarcinoma of the lung. Cancer Res. **54:** 5652–5655.
27. **Sozzi, G., Miozzo, M., donghi, R., Pilotti, S., Cariani, C.T., Pastorino, U., Porta, G.D. and Pierotti, M.A.** 1992. Detections of 17p and p53 mutation in preneoplastic lesions of the lung. Cancer Res. **52:** 6079–6082.
28. **Sozzi, G., Miozoo, M., Donghi, R., Pastorino, U., Piloti, S., Giarola, M., De Gregorio, L.D., Manenti, G., Radice, P., Minoletti, F., Porta, G.D. and Pierotti, M.A.** 1995. Genetic evidence for an independent origin of multiple preneoplastic and neoplastic lung lesions. Cancer Res. **55:** 135–140.
29. **Sundaresan, V., Gandy, P., Hasleton, P., Rudd, R., Sinha, G., Bleehen, N.M. and Rabbitts, P.** 1992. p53 and chromosome 3 abnormalities, charac-

teristic of malignant lung tumors, are detectable in preinvasive lesions of the bronchus. Oncogene **7:** 1989–1997.

30. **Suzuki, H., Takahashi, T., Suyama, M., Ariyoshi, Y., Takahashi, T. and Ueda, R.** 1992. p53 mutaions in non-small cell lung cancer in Japan: association between mutation and smoking. Cancer Res. **52:** 734–736.
31. **Takahashi, T., Nau, M.M., Chiba, I., Birrer, M.J., Rosenberg, R.K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A.F. and Minna, J.D.** 1989. p53: a frequent target for genetic abnormalities in lung cancer. Science **246:** 491–484.
32. **Tuchiya, E., Nakamura, Y., Weng, S.Y., Nakagawa, K., Tsuchiya, S., Sugano, H. and Kitagawa, T.** 1992. Allelotype of non-small cell lung carcinoma-comparison between loss of heterozygosity in squamous cell carcinoma and adenocarcinoma. Cancer Res. **52:** 2478–2481.
33. **Thiberville, L., Rayne, P., Vielkind, S.J., Leriche, J., Horsman, D., Nouvet, G., Palcic, B. and Lam, S.** 1995. Evidence of cumulative gene losses with progression of premalignant epithelial lesions to carcinoma of the bronchus. Cancer Res. **55:** 5133–5139.
34. **Walker, C., Robertoson, L.J., Myskow, M.W., Pendleton, N. and Dixon, G.R.** 1994. p53 expression in normal and dysplastic bronchial epithelium and in lung carcinoma. Br. J. Cancer. **70:** 297–303.
35. **Washimi, O., Nagata, M., Osada, H., Ueda, R., Koshikawa, T., Seki, T., Takahashi, T. and Takahashi, T.** 1995. In vivo occurrence of p16 (MTS1) and p15 (MTS2) alteration preferentially in non-small cell lung cancers. Cancer Res. **55:** 514–517.
36. **Lydiatt, W.M., Anderson, P.E., Bazzana, T., Casale, M., Hughes, C.J., Huvos, A.G., Lydiatt, D.D. and Schantz, S.P.** 1998. Molecular suppor for field cancerization in the Head and Neck. Cancer **82:** 1376–1380.
37. **Wright, D.K. and Mannos, M.M.** 1990. PCR Protocols: A guide to method and applications. pp. 153–158. Academic Press: San Diego.
38. **Yokota, J., Wada, M., Shimosato, Y., Terada, M. and Sugimura, T.** 1987. Loss of heterozygosity on chromosomes 3, 13, and 17 in small-cell carcinoma and on chromosome 3 in adenocarcinoma of the lung. Proc. Natl. Acad. Sci. **84:** 9252–9256.