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Detection of c-Ki-*ras* gene mutation in paraffin sections of adenocarcinoma and atypical bronchioloalveolar cell hyperplasia of human lung

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Abstract DNA of minute specimens taken from formalin-fixed and paraffin-embedded tissue was used as a template for the polymerase chain reaction (PCR) to examine whether the c-Ki-*ras* gene is activated in atypical bronchioloalveolar cell hyperplasia (ABH) of human lung. The c-Ki-*ras* gene was successfully amplified on 131 samples from 29 cases of lung adenocarcinoma (87.3% of all samples used as templates) by the nested PCR method. Point mutations at codon 12 of the c-Ki-*ras* gene could be detected in carcinoma tissue of 6 cases (20.7% of all cases), also detected in ABHs showing moderate to severe atypia of 2 cases. PCR amplification is a useful technique for studying pin-point pathological lesions in a routine paraffin section at the molecular level.

Key words Lung neoplasms · Precancerous conditions
Ki-*ras* genes · DNA mutational analysis
Polymerase chain reaction

Introduction

Focal atypical proliferation of bronchioloalveolar epithelial cells in the peripheral part of the lung has attracted attention as a possible precursor of adenocarcinoma (Kodama et al. 1986; Miller et al. 1988; Shimizu 1990; Weng et al. 1992). This lesion is called atypical bronchioloalveolar cell hyperplasia (ABH) (Weng et al. 1992) and known by other terms such as metaplasia with atypical changes (Shimizu 1990), glandular neoplasia (Miller et al. 1988) and atypical alveolar cuboidal cell hyperplasia (Kodama et al. 1986). Activation of oncogenes or inactivation of anti-oncogenes have been reported to be involved in the occurrence of lung adenocarcinoma (Buchhagen 1991; Reynolds and Anderson 1991; Rodenhuis and Slebos 1992) and genetic analysis on ABH is indicated. However, no genetic analyses in ABH at the molecular level have been reported; such lesions are usually so minute that they are hardly discernible on gross examination. In this study, the presence of point mutations of the c-Ki-*ras* gene was investigated by polymerase chain reaction (PCR) in minute samples of ABH that were specified by microscopic examination of formalin-fixed paraffin-embedded histological sections.

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Materials and methods

Lobectomy specimens of primary lung adenocarcinomas of 29 patients were used in this study obtained at Saitama Medical School Hospital and related hospitals during the period from 1984 to 1991. Primary lesions of all cases were solitary, whereas intrapulmonary metastases were found in three cases and bone metastasis was found in one case. ABH tissues were generally contiguous with the carcinoma tissue but some solitary lesions were also found. Lungs were infused with 10% formalin solution transbronchially and fixed for 4 days. Tissue samples were dehydrated and embedded in paraffin. Serial 4 μm and 10 μm sections were cut from paraffin blocks and mounted on glass slides. The 4 μm sections were stained with haematoxylin-eosin and studied microscopically to determine sampling area for PCR analysis.

ABH and adenocarcinoma were defined histologically as follows; ABH-focal proliferating lesions in the periphery of the lung consisting of cuboidal or round cells with various degrees of atypia, arranged on alveolar septa. The degree of atypia was classified into mild, moderate and severe by the variation in nuclear and cell size, nuclear polymorphism, and cellularity. Adenocarcinoma was defined as proliferating lesions consisting of cells with more marked atypia than ABH, often characterized by cellular piling up.

The 10 μm sections were deparaffinized and lightly stained with haematoxylin, and the tissues of previously selected areas were scraped from semi-dried sections with the point of a sterile toothpick under the stereomicroscope. The tissue sample stuck to the toothpick was transferred into a sterile 0.5 ml microtube. Between 10 and 50 μl of digestion buffer (200 μg/ml proteinase K, 0.5% Tween 20, 1 mM EDTA, 10 mM TRIS buffer, pH 8.0) was added to each tube and incubated at 42° C for 2 days, and 200 μg/ml of proteinase K was added halfway through incubation. After incubation, proteinase K was heat-inactivated at 95° C for

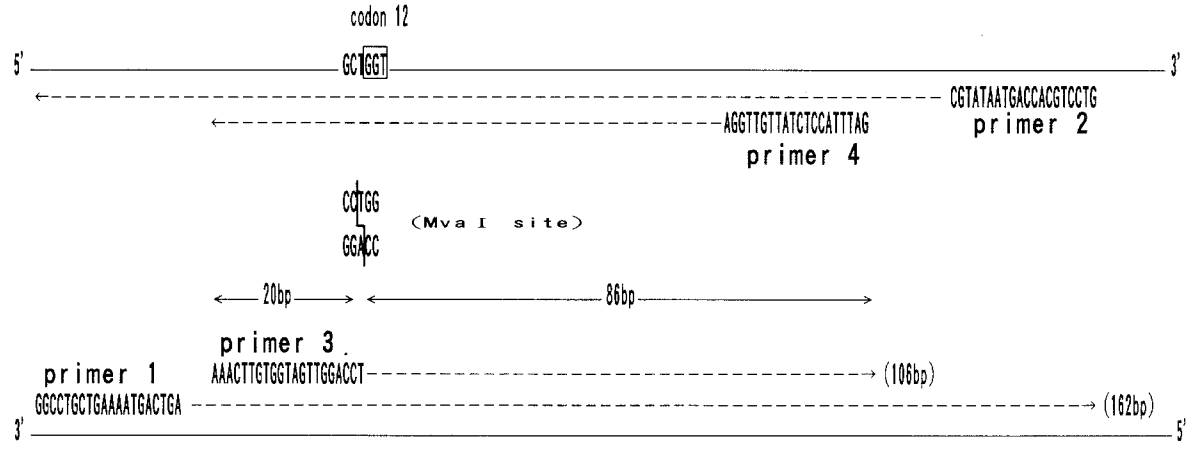

Fig. 1 Strategy for detection of point mutations in codon 12 of the human c-Ki-*ras* gene using nested polymerase chain reaction (PCR) and designed restriction fragment length polymorphism (RFLP). First PCR with primer 1 and primer 2 amplifies the 162 bp fragment including codon 12. Second PCR with primer 3 and primer 4 amplifies the 106 bp fragment, and introduces Mva I site since primer 3 contains a mismatch in codon 11 (GCT→CCT) for creation of a sequence CCTGG. The product of the second PCR is digested with Mva I, so that wild type DNA is digested to generate 86 and 20 bp fragments, while mutant DNA is not digested. Undigested DNA is detected by electrophoresis and Southern blotting

15 min, and 10 µl of digested sample was used as a template for PCR.

A sequence including codon 12 of the c-Ki-*ras* gene was amplified by the nested PCR method using two sets of primers (Fig. 1). Primers for the first PCR were those reported by other investigators (Suzuki et al. 1990) and primers for the second PCR were designed originally to detect the mutation of codon 12 of c-Ki-*ras* gene. First PCR reactions were performed in 50 µl reaction mix containing 50 mM potassium chloride, 1.5 mM magnesium chloride, 10 mM TRIS-HCl (pH 8.3), 0.001% gelatin, 200 µM of each nucleotide (dATP, dTTP, dCTP, dGTP), 0.5 µM of each primer, 1 unit of *Taq* DNA polymerase (Perkin-Elmer Cetus), and 10 µl of template. After an initial 5 min denaturation at 94° C, 30 cycles of 94° C for 1 min, 57° C for 1 min, 72° C for 1 min, were carried out, followed by a 7 min extension at 72° C using the DNA Thermal Cycler (Perkin-Elmer Cetus). The product of the first PCR was diluted 1:100 in TRIS-EDTA buffer (10 mM TRIS-HCl pH 7.5, 1 mM EDTA) and 5 µl of the dilution was used as the template for a second PCR. Second PCR reactions were performed under the same condition as the first PCR, except that annealing was carried out at 55° C. In each experiment, three control reactions, containing no template DNA (negative control), normal DNA extracted from normal human tissue (normal control) and mutation-positive DNA from human lung cancer cell line A549 (positive control), were always conducted simultaneously with sample reactions. The final reaction mixture (10 µl) was electrophoresed in 2.5% agarose gel and stained with ethidium bromide to confirm the success of amplification. When amplification did not take place in a sample, proteinase K treated template was purified by phenol-chloroform extraction and PCR was re-attempted. The amplified sample (8 µl) was digested with 10 u of Mva I at 37° C overnight and electrophoresed in 2.5% agarose gel to determine the presence of mutations at codon 12. After electrophoresis, the DNA fragments were transferred onto nylon membrane (Magnagraph, MSI Co.) to detect small amounts of mutated DNA in the sample. Uncut DNA was detected with a mixture of six oligonucleotide probes capable of hybridizing to all sequences mutated at codon 12, using an ECL-3′ oligolabelling and detection system (Amersham). This hybridization allowed detection of 2% mutant DNA contained in an amplified DNA. In some cases, dot blot hybridization with oligonucleotide probes was carried out to identify base substitutions in the samples that showed positive results by restriction fragment length polymorphism (RFLP) analysis described above. In this hybridization, oligonucleotides were labelled with a Dig Oligonucleotide Tailing Kit and signals were detected with a Dig Luminescent Detection Kit (Behringer Mannheim Biochemica). Primers and probes were synthesized by Cyclon Plus DNA Synthesizer (Milligen/Biosearch, Division of Millipore). To avoid contamination, reaction mixes for PCR were prepared in a laminar flow hood after the inside of the hood had been exposed to UV light. Different sets of pipettes were used for preparation of pre-PCR materials and for analysis of amplified DNA. Disposable gloves were worn and changes frequently. Reagents and equipment, such as buffers, distilled water, pipette tips, microtubes were autoclaved.

Results

In this study, point mutations at codon 12 of the c-Ki-*ras* gene in tissue samples from routine paraffin sections as small as 1 mm in diameter could be investigated successfully. Figure 2 shows typical results of electrophoresis and Southern blotting. In this case, point mutation was detected in adenocarcinoma tissue and in "ABH with severe atypia", but not in "ABH with mild atypia" or normal tissue. Figure 3 shows examples of adenocarcinoma tissue and ABHs that were scraped off and analysed for the presence of mutations at codon 12 of the c-Ki-*ras* gene. This case corresponds to the case shown in Fig. 2. Adenocarcinoma tissue and "ABHs with severe atypia" in this case had the same mutation (GGT→GCT) while "ABHs with mild atypia" and normal tissues had no mutations.

In the present study, 150 samples from 29 patients, including 100 samples of adenocarcinoma tissue from 29 patients, 20 samples of ABHs from 6 patients, and 30 samples of normal tissue from 21 patients were used as templates for the PCR, and 131 samples from 29 patients (87.3% of all samples) were successfully amplified and analysis of point mutations at codon 12 of the hu-

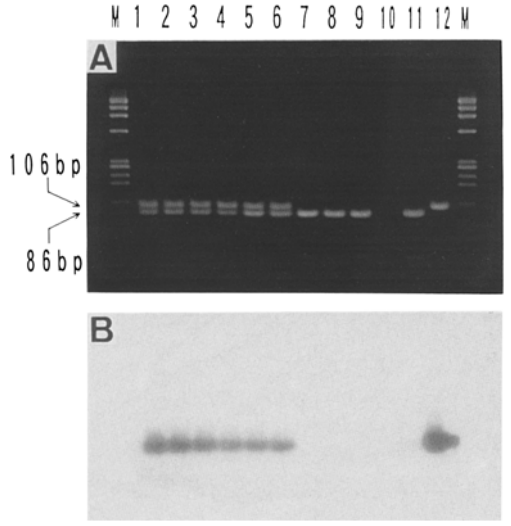

Fig. 2A, B RFLP analyses by electrophoresis (**A**) and Southern blotting (**B**). Mva I digests from adenocarcinoma tissue (lane 1, 2) and atypical bronchioloalveolar hyperplasia (ABH) with severe atypia (lane 3–6) show both normal and mutated DNA (106 bp and 86 bp), while those from ABH with mild atypia (lane 7, 8) and normal tissue (lane 9) show normal DNA only. The negative control (lane 10) shows no bands, the normal control (lane 11) shows normal DNA only and the positive control (lane 12) shows mutated DNA only. Southern blotting reveals hybridization signals of undigested DNA

man c-Ki-*ras* gene was possible (Table 1). In 14 samples, PCR amplification was successful after phenol-chloroform extraction of proteinase K-treated templates.

Point mutations at codon 12 of the c-Ki-*ras* gene were detected in 19 samples of cancer tissue from 6 patients out of 86 samples of cancer tissue from 29 patients (Table 2). Age, sex, stage of the tumour, site of the tumour, histological subtype, degree of differentiation, and mutation frequency of each case are shown in Table 3. Average age of mutation positive cases was 62.3 and that of negative cases was 64.0 years old respectively, and the difference was not significant (*t*-test). The ratio of male to female number was 2.0 (4 cases:2 cases) in positive cases and 1.56 (14 cases:9 cases) in negative cases; this difference was not significant in the chi-square test. Although tumour stages tended to be less advanced in positive cases when compared with negative cases, the difference was not significant in the chi-square test since cases were few. Histological subtypes were also nearly the same in both cases as shown in Table 3. Although the degree of differentiation in mutation positive cases seemed to be somewhat higher than negative cases, the difference was not significant in chi-square test. Moreover, the incidence of ABH seemed to be rather high in mutation positive cases when compared with negative cases, but was also not significant in the chi-square test.

Mutation was also detected in 9 ABHs from 2 patients out of 18 ABHs from 6 patients. These 9 samples included 7 ABHs exhibiting severe atypia, one ABH with moderate atypia in case 4, and one ABH with severe atypia in case 2. No mutations were detected in 27 samples of normal tissue from 19 patients.

Discussion

Although the pathogenesis of primary adenocarcinoma of the human lung remains unclear, lesions such as ABH have been discussed by several investigators as possible precursor lesions of adenocarcinoma (Kodama et al. 1986; Miller et al. 1988; Shimizu 1990; Weng et al. 1991). However, these discussions are principally based on morphological observations; there is no conclusive evidence about the relationship between ABH and adenocarcinoma. Since recent progress in molecular biology has clarified that the development of neoplasms is

Table 1 Success rate of polymerase chain reaction (PCR) amplification

Tissue	Number of samples (cases) used as templates	Number of samples (cases) amplified by PCR	Success rate of PCR
			Samples (cases)
Normal tissue	30 (21)	27 (19)	90.0% (90.5%)
ABH tissue	20 (6)	18 (6)	90.0% (100%)
Carcinoma tissue	100 (29)	86 (29)	86.0% (100%)
Total	150 (29)	131 (29)	87.3% (100%)

Table 2 Frequency of point mutations in codon 12 of the c-Ki-*ras* oncogene

Tissue	Number of samples (cases) amplified by PCR	Number of samples (cases) with mutation	Frequency of mutation
			Samples (cases)
Normal tissue	27 (19)	0 (0)	0.0% (0.0%)
ABH tissue	18 (6)	9 (2)	50.0% (33.3%)
Carcinoma tissue	86 (29)	19 (6)	22.1% (20.7%)
Total	131 (29)	28 (6)	21.4% (20.7%)

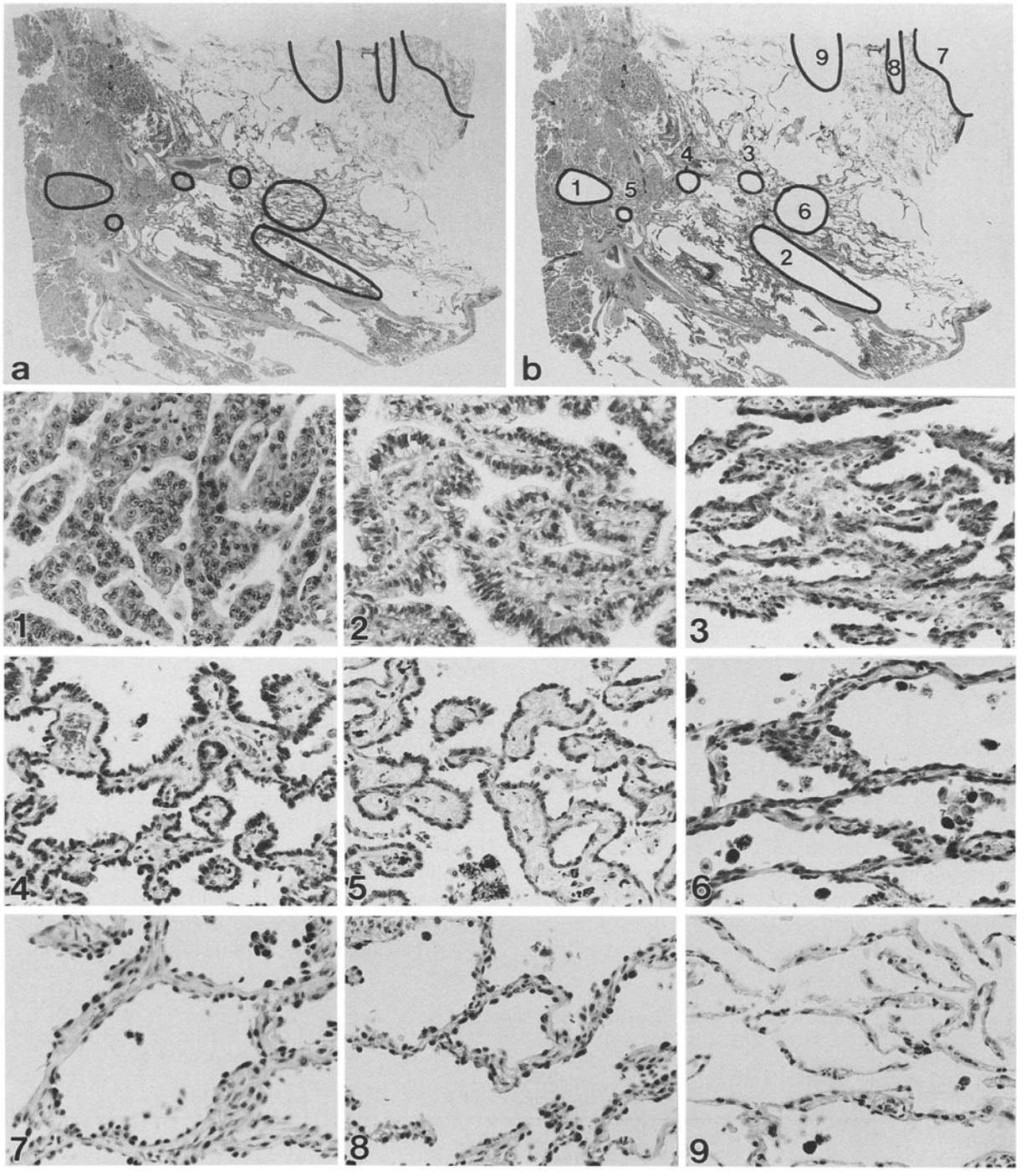

Fig. 3a, b An example of adenocarcinoma tissue and ABH which had been scraped and analysed for the presence of mutation. **a** Neighboring section with scraped one. H&E, ×2.7. **b** After scraping for PCR. H&E, ×2.7. **1~9** High magnification views of each area indicated in **a, b** evaluated as adenocacinomas (1, 2). ABHs with severe atypia (3–6), ABHs with mild atypia (7, 8) and normal tissues (1, 2). H&E, ×157. In this case, area 1 to 6 had the same mutation (GGT→GCT) at codon 12, while area 7 to 9 had no mutation. This case corresponds to the case shown in **Fig. 2** and areas 1–9 correspond to lanes 1–9 in **Fig. 2**

closely related to structural changes in some oncogenes and anti-oncogenes (Fearon and Vogelstein 1990; Lehman et al. 1991; Wynford-Thomas 1991), it is expected that the biological significance of ABH could be elucidated by investigating genetic changes in the lesion. Such studies have not been reported so far, since the lesions are usually so minute that they are hardly discernible on gross examination. It seems to be important to establish a method that enables us to analyse DNA from minute specimens, identified microscopically, using reoutinely processed histological sections.

Table 3 Clinical and histological data of studied cases and mutation frequency in each focus (*L* left lobe; *R* right lobe; *U* upper lobe; *L* lower lobe; *P* papillary type; *T* tubular type; *b–a* bronchiolo-alveolar type; *W* well; *M* moderate; *P* poor)

No.	Sex	Age	Stage[a]	Tumor Site	Subtype	Degree of differentiation	Mutation frequency[c]				
							Normal tissue	ABH[d]			Carcinoma tissue
								Mild	Moderate	Severe	
1	M	69	I	LU	P+T	W	0/1		0/1[e]		1/2
2	M	52	IIIA	RU	P	W				1/2	3/5
3	F	54	I	RU	P(b–a)	W					1/1
4	M	71	I	RU	P	M~W	0/5	0/4[e]	1/1	7/7	9/9
5	F	48	I	LU	P	P~W	0/2				3/19
6	M	80	I	LL	P	M	0/1				2/2
7	F	55	IV	RL	P	W	0/1			0/1[e]	0/3
8	F	63	I	RM	P	W	0/1			0/1[e]	0/1
9	M	61	I	LU	P	W	0/2				0/1
10	M	73	I	RL	P	W					0/2
11	M	53	II	RU	P	W	0/1				0/2
12	F	75	IIIA	RL	P	W	0/1				0/1
13	F	43	I	RU	P	W	0/1				0/2
14	M	75	IIIA	LL	P	M	0/1			0/1	0/3
15	F	71	II	LL	T	M~W	0/1				0/3
16	M	71	II	LL	P	M~W					0/1
17	M	56	II	RL	P	M~W	0/1				0/2
18	F	49	I	LL	P	P+W	0/1				0/4
19	F	72	IV	RU(+M)	P	P~W	0/1				0/3
20	M	50	IIIA	RL	P	M					0/2
21	M	72	IIIA	RU	P	M	0/2				0/4
22	M	61	I	RU	P	M					0/1
23	F	64	I	LL	P	M					0/1
24	M	78	IIIA	RU	P	M	0/1				0/2
25	M	77	I	RM	P(b–a)	M	0/2				0/4
26	M	68	II	RU	P	M	0/1				0/2
27	F	43	IV	RU	T	P	0/1				0/2
28	M	76	II/IIIA[b]	RL	T	P					0/1
29	M	65	IV	RU	P	P					0/1
Total							0/27	0/4	1/2	8/12	19/86
									9/18		

[a] According to "General Rule for Clinical and Pathological Record of Lung Cancer" edited by The Japan Lung Cancer Society.
[b] Location of lymph node is unidentified.
[c] No. of positive samples / No. of amplified samples.
[d] Atypical bronchioloalveolar cell hyperplasia. [e] Separately found from primary carcinoma

The PCR method is a powerful technique to analyse DNA from minute samples (Mcpherson et al. 1991) and several investigators have applied PCR method to analysis of DNA using paraffin-embedded tissue (Rogers et al. 1990). However, tissues fixed in formalin for long periods are not always suitable for PCR templates, and low efficiencies of amplification have often been encountered (Rogers et al. 1990). We overcame low efficiency by the use of the nested PCR method, which is reported to be highly sensitive and specific (Jackson et al. 1991). Consequently, we achieved a high success rate using minute specimens taken from paraffin section as templates. Nearly 90% of templates from routine processed operation materials could be amplified. Some specimens, however, failed to be amplified probably because DNA in the tissue had been damaged by processing and formalin fixation. The RFLP method employed in this study to detect c-Ki-*ras* gene mutation has been used by several investigators formerly (Kumar and Dunn 1989). The method is not only very convenient but also highly sensitive if it is used combined with southern blotting method as in our study. We only examined mutations at codon 12 of c-Ki-*ras* gene but this method could be applied to other loci such as codon 13 or codon 61 of the same gene by use of specific primers designed for RFLP.

The significance of ABH in the pathogenesis of primary adenocarcinoma of the human lung is a matter of controversy. A high incidence of ABH in cases of adenocarcinoma has been reported by several investigators (Shimizu 1990; Weng et al. 1992), but the neoplastic nature of ABH and the clonal identity of the adenocarcinoma tissue and ABH has not been demonstrated by any method. In our study, it was shown that ABH and carcinoma tissue in case 4 have the same type of point mutation in the c-Ki-*ras* gene. The data indicates that ABH of case 4 may be involved in neoplastic pro-

cess of the adenocarcinoma. This situation resembles that of colorectal adenoma and colorectal carcinoma, which is reported to have the same type mutations in the c-Ki-*ras* gene (Bos et al. 1987; Forrester et al. 1987; Baker et al. 1990). However, the possibility that the lesion is a peripheral extension or peripheral invasion of the carcinoma cannot be ruled out by the data on c-Ki-*ras* gene only. Therefore, it seems to be necessary to investigate plural changes including other genes such as p53, to determine precise chronological relationship between ABH and adenocarcinoma.

However the carcinomas in some cases showed inconsistent results concerning the presence of c-Ki-*ras* mutation. There were mutation positive areas and negative lesions in the same cases, possibly because mutation of the c-Ki-*ras* gene occurred during the evolution of the tumour although c-Ki-*ras* gene mutation is reported to be a relatively early event in the development of colon cancers (Baker et al. 1990; Fearon and Vogelstein 1990).

In summary, mutational change of c-Ki-*ras* gene in minute tissues of ABH taken from routine paraffin sections was successfully investigated by nested PCR method. Point mutations at codon 12 of the c-Ki-*ras* gene were detected in carcinoma and ABH tissues as small as 1 mm in diameter. ABHs in some cases were closely related to coexisting adenocarcinoma. Our method is useful technique for studying pin-point pathological lesions in a routine paraffin section at the molecular level.

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