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p16/CDKN2 Gene and *p53* Gene Alterations in Japanese Non-smoking Female Lung Adenocarcinoma

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Primary lung adenocarcinomas in non-smoking females are increasing in the USA and Japan. Environmental factors such as passive smoking, asbestos, domestic radon, and hormonal effects have been implicated, but the etiology is still uncertain. We therefore analyzed point mutations of *p16* gene, a newly characterized tumor suppressor gene, and compared the results with alterations of *p53* gene in 28 primary lung adenocarcinomas in non-smoking Japanese females. There were no cases with somatic point mutation of *p16* gene, except for one case with two germline mutations (silent mutations). In contrast, six out of 16 informative cases showed loss of heterozygosity of *p53* gene using a *TP53* microsatellite marker and 19 out of 28 cases showed expression of oncoprotein using DO-7 immunohistochemistry. These findings suggest that *p16* gene alteration is a rare event in primary lung adenocarcinomas in Japanese non-smoking females, compared with alterations of the *p53* gene.

Key words: *p16/CDKN2* — *p53* — Non-smoker — Lung adenocarcinoma — PCR-SSCP

The etiology of lung cancer in non-smokers has not yet been clearly ascertained. Many environmental carcinogens, including passive inhalation of tobacco smoke,[1] asbestos,[2] domestic radon,[3] and a hormonal effect[4] have been implicated in the occurrence of cancer by epidemiologic studies. In the USA, the proportion of adenocarcinomas among primary lung cancers is increasing,[5] while in Japan, adenocarcinoma has always been the most prevalent histologic type of primary lung cancer in females.[6] It is therefore necessary to identify the causative carcinogens and to ascertain their carcinogenic mechanisms.

Mutational spectra of the *p53* gene in cancer cells can be associated with the specific type of carcinogen.[7–9] For example, we have already reported that the spectrum of *p53* gene mutation in lung cancer in females is mainly G:C to A:T transition,[10] whereas in smokers, G:C to T:A transversion is frequent.[11] These findings suggest that carcinogens, such as benzo[*a*]pyrene in tobacco smoke, are responsible for the high proportion of G:C to T:A transversions and that the analysis of mutational spectra of oncogenes and/or suppressor genes, including K-*ras* and *p53* genes, may be useful in generating hypotheses regarding the putative potential carcinogens.

Recently, a new tumor suppressor gene, *p16/CDKN2* gene, was discovered on chromosome 9p21.[12, 13] *p16*[2] inhibits the binding of cyclin-dependent kinase 4 to cyclin D1[13, 14] and modulates the G1 checkpoint.[15] Reciprocal *Rb* inactivation and *p16* expression in primary lung cancers and their cell lines have also been reported.[15, 16] *p16* gene alterations, including homozygous deletions and point mutations, in human cancer have been observed in glioma,[17–22] esophageal carcinoma,[23–25] pancreatic carcinoma,[24, 26] malignant mesothelioma,[27] tumors of the head and neck,[28] familial melanoma,[29] breast carcinoma,[30] leukemia[31] and non-small cell lung carcinoma.[32–34] Some workers have pointed out that *p16* gene alterations in *in vivo* tumors are less frequent than in *in vitro* cell lines,[35, 36] and it has been speculated that the establishment of cell lines is more easily achieved using tumors with *p16* gene alterations.[36]

In order to ascertain the etiology of lung adenocarcinoma in non-smoking females, in the present study we have attempted to examine *p16* gene alterations in comparison with *p53* alterations.

MATERIALS AND METHODS

Case selection Twenty-eight cases of primary lung adenocarcinoma in non-smoking females during the period 1992–1994 were obtained from the files of the Department of Pathology, Hiroshima University School of Medicine, Japan. Clinical information and pathological features are summarized in Table I. Pathological classification of lung adenocarcinoma was confirmed according to the general rules for clinical and pathological records of lung cancer of the Japan Lung Cancer Soci-

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[2] Abbreviations: *p16*, *p16/CDKN2*; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism; LOH, loss of heterozygosity.

Table I. The Clinical and Pathological Features of Cases, and the Results on Gene Alterations

Case no.	Age	Subtype of tumor	Grade of differentiation	Stage	*p16* gene		9p21-22		*p53*	
					Exon 1	Exon 2	*IFNA*	*D9S171*	Expression	*TP53*
FT1	56	Pap	mod	I	WT	WT	NI	NLOH	−	LOH
FT2	46	Pap	well	I	WT	WT	NI	NI	+++	LOH
FT3	61	Pap	mod	IIIA	WT	WT	NLOH	NI	+++	LOH
FT4	61	Pap	well	I	WT	WT	LOH	NLOH	++	NLOH
FT5	68	Pap	well	I	WT	WT	NI	NA	+	NI
FT6	59	Pap	mod	IIIA	WT	WT	NI	NI	−	NLOH
FT7	43	Pap	mod	I	WT	WT	NI	NI	+++	LOH
FT8	51	Pap	mod	IV	WT	Germ[a)]	NLOH	NLOH	+	NLOH
FT9	50	Pap	mod	IIIA	WT	WT	NLOH	NI	+++	NI
FT10	58	Pap	mod	I	WT	WT	NLOH	NI	+++	LOH
FT12	63	Pap	well	I	WT	WT	NI	NI	+++	NLOH
FT13	70	Tub	poor	I	WT	WT	NI	NI	++	NLOH
FT14	74	Pap	mod	IIIA	WT	WT	NLOH	NI	+	LOH
FT15	77	Pap	mod	I	WT	WT	NI	NI	+	NLOH
FT16	64	Pap	well	I	WT	WT	NLOH	NI	+	NLOH
FT17	61	Pap	mod	I	WT	WT	NLOH	LOH	−	NLOH
FT18	56	Pap	well	I	WT	WT	NLOH	NLOH	−	NLOH
FT20	71	Tub	well	I	WT	WT	NI	NI	+	NLOH
FT21	58	Pap	well	I	WT	WT	ND	ND	−	ND
FT22	75	Pap	well	I	WT	WT	ND	ND	−	ND
FT23	80	Pap	well	I	ND	ND	ND	ND	++	ND
FT24	43	Tub	mod	IIIA	ND	ND	ND	ND	−	ND
FT25	76	Pap	mod	IV	ND	ND	ND	ND	+	ND
FT26	72	Pap	well	I	ND	ND	ND	ND	+	ND
FT27	62	Pap	well	I	ND	ND	ND	ND	++	ND
FT28	58	BR-AL	well	I	ND	ND	ND	ND	−	ND
FT29	54	Tub	mod	IIIA	ND	ND	ND	ND	+	ND
FT30	64	BR-AL	well	I	ND	ND	ND	ND	−	ND
Total					0/20	0/20	1/9	1/5	19/28	6/16

Pap, papillary type; Tub, tubular type; BR-AL, bronchiolo-alveolar type; well, well differentiated; mod, moderately differentiated; poor, poorly differentiated; LOH, loss of heterozygosity; NLOH, no loss of heterozygosity; NI, not informative; ND, not done; NA, not amplified.

a) Germline mutation: codon 75, CAC to CAT, codon 76, GAC to GAT (both are silent mutations).

ety.[37] Precise details on passive smoking, and environmental and occupational exposure to specific carcinogens were not available.

DNA extraction Lung tissues were fixed with formalin and embedded in paraffin wax. Two paraffin blocks with lung tumor tissue and non-neoplastic peripheral lung tissue, were prepared for each patient. Sections 10 μm thick were cut from each block and microtome blades were changed between blocks to prevent tissue carryover. Tissue sections were dewaxed using xylene and 100% ethanol. Well-preserved tumor- and non-tumor-containing tissues were dissected from the 10 μm sections using sterile needles. At least 50% of the cells in the scraped area were carcinoma cells. Genomic DNA was isolated using standard methods.[38, 39]

PCR-SSCP and sequencing analyses of *p16* gene Exon 1 and exon 2 of *p16* gene were amplified from tumor- and non-tumor-bearing tissue DNA using specific primers of *p16* gene as described previously. Exon 2 included three overlapping PCR products,[29] designated "the first region," "the middle region" and "the last region." The primer sequences of exon 1 and these three regions of exon 2 are as follows (5′–3′), exon 1: GGGAGCAGCATGGAGCCG and AGTCGCCCGCCATCCCCT; the first region of exon 2: AGCTTCCTTTCCGTCATGC and GCAGCACCACCAGCGTG; the middle region of exon 2: AGCCCAACTGCGCCGAC and CCAGGTCCACGGGCAGA and the last region of exon 2: TGGACGTGCGCGATGC and GGAAGCTCTCAGGGTACAAATTC. PCR was carried out using 2 μl of genomic DNA (100 ng), 2.5 μl of 10× buffer (1×10 m*M* Tris (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin), 2 μl of dNTP (1.25 m*M* of each dATP, dCTP, dGTP and dTTP), 0.5 μl of forward primer (50 μM) and 0.5 μl of

reverse primer (50 μM), 0.25 μl of Taq polymerase (5 U/μl) and 0.2 μl of [α-^{32}P]dATP. For amplification, each sample was denatured at 94°C for 5 min, and subjected to 40 amplification cycles. The annealing temperature of each primer set was 58°C. After PCR, 5 μl of PCR product was diluted with 95 μl of denaturing loading buffer and denatured at 94°C for 3 min, then 3 μl samples were electrophoresed on 6% non-denaturing polyacrylamide gel at 10 W for 5 h at 4 and 20°C, with and without glycerol. After electrophoresis, polyacrylamide gels were dried and exposed to X-ray film overnight. When variant SSCP bands were detected, the PCR products were sequenced directly using a "Sequenase" PCR-products sequencing kit (USB-Amersham, Cleveland, OH). We also sequenced the PCR products directly from 10 adenocarcinoma samples (FT1–FT10) to confirm the sensitivity of the PCR-SSCP methods.[40]

Analysis of LOH on 17p13 and 9p21-22 using microsatellite markers To detect allelic loss on 17p13 and 9p21-22, markers including *TP53*,[41] *IFNA*[42] and *D9S171*[43] were used. PCR amplification of polymorphic CA repeats was carried out in essentially the same way as described previously.[44] The primer sequences of microsatellite markers are as follows (5′–3′), *TP53*: CCCCATTCCCCTTTCCCT and ACTATTCAGCCCGAGGTGC, *IFNA*: TGCGCGTTAAGTTAATTGGTT and GTAAGGTGGAAACCCCCACTPCR and *D9S171*: CCCTAGCACTGATGGTATAGTCT and GCTAAGTGAACCTCATCTCTGCT. PCR products were electrophoresed on 6% denaturing polyacrylamide gel. For informative cases, allelic loss was defined as having occurred if the intensity of the autoradiographic signal of a given allele was reduced by at least 50% in the tumor DNA as compared with that of the corresponding normal allele.[45]

Immunohistochemistry The monoclonal antibody DO-7 (Novocastra, Newcastle, UK), which reacts with both wild-type and mutant forms of *p53*, was used for immunohistochemistry. Formalin-fixed 5 μm sections were cut from each block. The avidin-biotin-peroxidase complex method with an antigen retrieval procedure was used[46] with DO-7 antibody diluted 1:50. The staining pattern was scored, according to the percentage of tumor cells which were stained as follows: +++ (marked; more than 70% tumor cells were stained intensely), ++ (moderate; 30–70% stained intensely), + (mild; less than 30% stained, intensely or weakly stained); or − (no staining).

Statistical analysis The correlation between the status of *p53* alteration including *p53* immunopositivity and LOH of *TP53*, and the clinical and pathological parameters (i.e., age, histological subtype, tumor differentiation and stage) were analyzed by using the chi-square method and Fisher's exact test.

RESULTS

PCR-SSCP and direct sequencing analyses of *p16* gene Out of the 20 cases examined by PCR-SSCP analysis, none showed abnormally shifted bands in exon 1 or the first and last regions of exon 2. In the middle region of exon 2, one case (FT8) showed an abnormally shifted band (Fig. 1). When the PCR products from normal tissue were electrophoresed on the same gel, the same abnormally shifted bands were observed (data not shown). Using direct sequencing, this case showed mutations on codon 83, CAC to CAT (His to His) and codon 84, GAC to GAT (Asp to Asp) in tumor tissue as well as in non-neoplastic tissue. These were silent mutations (Fig. 2).

LOH on 9p21-22 locus One case (FT4) showed LOH in the *IFNA* locus, and another (FT17) did so in *D9S171* (Fig. 3). No other informative cases showed LOH.

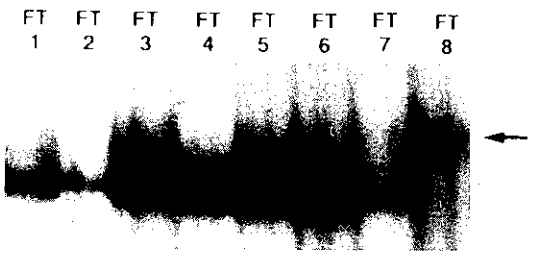

Fig. 1. PCR-SSCP analysis of exon 2 of *p16* gene, middle region. An abnormally shifted band was noted in case FT8 (arrow).

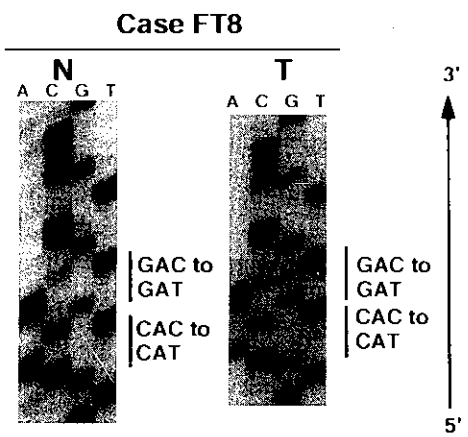

Fig. 2. Direct sequencing of *p16* gene. Case FT8 showed the same silent mutations on codon 83 and codon 84, CAC to CAT (His to His), GAC to GAT (Asp to Asp) in tumor tissue and non-neoplastic tissue.

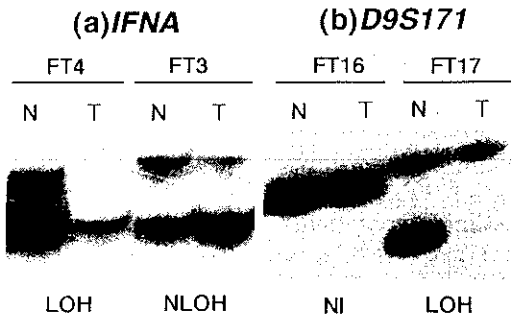

Fig. 3. LOH analysis of microsatellite marker on 9p21-22. (a) Case FT4 showed LOH on *IFNA* locus, but FT3 showed no LOH. (b) Case FT17 showed LOH in *D9S171* locus, and case FT16 was not informative.

Fig. 4. LOH analysis on *TP53* locus. Case FT3 showed LOH, but case FT4 showed no LOH.

LOH on 17p and immunohistochemistry of the oncoprotein of *p53* gene Using a microsatellite marker of *TP53* locus, six out of 16 informative cases showed LOH, but no microsatellite instability was found (Fig. 4). As for the immunohistochemistry of *p53*, six cases with marked reactivity (Fig. 5), four cases with moderate reactivity, nine cases with mild reactivity and nine others with negative staining were observed.

Comparing LOH and *p53* immunopositivity, four out of six cases with LOH showed marked positivity, one showed mild positivity and one case was negative. Out of 10 cases without LOH, one showed marked positivity, two moderate positivity and four mild positivity, with the remaining three cases showing negative staining.

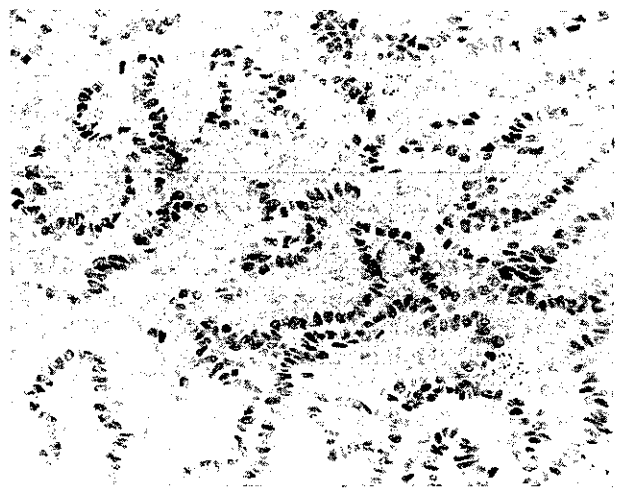

Fig. 5. Immunohistochemistry of *p53* oncoprotein. Almost all carcinoma cells showed strong positivity for DO-7 antibody.

No significant correlation between *p53* abnormalities, and any clinical and pathological parameters was observed among the present cases (data not shown).

DISCUSSION

In this study, we did not detect any mutation in *p16* gene in lung adenocarcinoma from non-smoking females using PCR-SSCP analysis. This implies that point mutation of *p16* might be a rare event in primary lung adenocarcinoma. Among the previous reports, Hayashi *et al.*[32] reported that somatic mutations of *p16* gene were observed in 19 out of 64 primary non-small cell lung cancers (10 out of 31 adenocarcinomas and 9 out of 33 squamous cell carcinomas), while Okamoto *et al.*[34] found no somatic mutations and no homozygous deletions in 25 primary non-small cell carcinomas and 15 primary small cell carcinomas. This discrepancy may reflect differences in clinical background (i.e., smoking history, sex, age, histologic subtype and stage) in these reports. Okamoto *et al.*[34] reported that *p16* gene mutations of lung carcinomas are more frequent in metastatic carcinoma than in primary carcinoma.

Alternatively, it has been suggested that homozygous deletion of *p16* gene is the main alteration, instead of point mutation. In order to detect homozygous deletions, it is preferable to use cell lines or tumor tissues which contain small amounts of non-neoplastic cells. In this study, we carried out microdissection using a dissection microscope in order to exclude non-neoplastic cells, but it appeared that non-neoplastic cells could not have been completely excluded. Though each exon of *p16* gene

was amplified efficiently by PCR in every case, homozygous deletions could not be excluded in these lung cancers. Further investigation using Southern blot analysis is necessary. In one case (FT8), germline mutations were detected. These have not been reported previously, and it cannot be ruled out that they are either a new disease-associated mutation or a rare form of polymorphism.

We found two cases with LOH where the microsatellite marker was located close to the *p16* gene (*IFNA* and *D9S171*). This incidence was lower than reported previously.[44] These findings might suggest that the 9p21-22 locus close to the *p16* gene is not a primary target in lung adenocarcinoma in non-smoking Japanese females. This result should be extended by using many microsatellite markers within this region.

On the other hand, alterations of *p53* gene (oncoprotein overexpression and LOH of *TP53* locus) were frequently observed in these cases. The incidence is relatively higher than in adenocarcinoma in American non-smokers.[47] Dosaka-Akita *et al.* reported no cases of *p53* immunopositivity in lung adenocarcinoma in Japanese females.[48] This finding may reflect differences in carcinogens between the USA and Japan, and between different areas of Japan. Comparative analyses of the *p53* gene are necessary to reach definite conclusions.

It has been suggested that there is a close correlation between the presence of missense mutation and immunopositivity of *p53* gene.[49] Comparison of the LOH and the immunohistochemical results for *p53* showed a close relationship, except in a few cases. These findings suggest that *p53* immunohistochemistry is not always correlated with *p53* gene alterations, and in some cases wild-type protein is expressed.[50] Sequence analysis of *p53* gene is thus necessary.

In summary, alteration of the *p16* gene is a rare event in primary lung adenocarcinomas of non-smoking Japanese females, compared with *p53* alterations. It will be necessary to analyze other target genes to understand the etiology of these cancers.

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