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High Frequency of p53 Abnormality in Laryngeal Cancers of Heavy Smokers and Its Relation to Human Papillomavirus Infection

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A series of 41 laryngeal squamous cell carcinomas was examined for p53 abnormalities and human papillomavirus (HPV) infection by an immunohistochemical and/or molecular approach. Immunohistochemically, p53 over-expression was observed in about 60% of the cancers, of which 12 were revealed to contain point mutations of p53 by a combination of the single-strand conformational polymorphism technique and direct sequencing. The p53 point mutations ranged from codons 157 to 278 and most of these mutations lay in two "hot spots" (codon 157 in four cancers and codon 248 in three cancers). The majority of p53 mutations, both transversions (seven cancers) and transitions (five cancers), occurred at the G nucleotide of the codons. An analysis of the clinical information indicated that p53 point mutation was frequently observed in heavy smokers with an average Brinkman index score of more than 1000. On the other hand, HPV DNA, type 16 or 18, was detected in a quarter of the laryngeal cancers. Of eleven HPV-positive cases, nine were immunohistochemically positive for p53, of which four contained a p53 point mutation. These results suggested no inverse relation between p53 mutation and HPV infection in laryngeal cancers. Our study indicates that p53 abnormalities are related to smoking history and the correlation might be better for smoking and chemical mutagenesis than for HPV.

Key words: p53 — Immunohistochemistry — Point mutation — HPV — Laryngeal cancer

The nuclear phosphoprotein p53 was originally discovered to form an oligomeric complex with SV40 large T antigen in SV40-transformed cells.[1,2] Later, it became evident that p53 is a cell growth regulator acting as a tumor suppressor.[3] In humans, p53 (located on chromosome 17p) has been examined in a wide variety of tumors.[4] Various abnormalities of p53 including allelic loss, deletion and point mutation are believed to be the most common alterations in human cancers, so that the inactivation of wild-type p53 is considered to be a key event in the development of human cancers.[5,6]

In laryngeal cancers, p53 abnormalities such as over-expression, allelic loss and point mutation have been most frequently observed among various abnormalities of many oncogenes and antioncogenes, and are known to contribute to neoplastic transformation of laryngeal epithelium.[7–12] Furthermore, p53 over-expression or mutation in laryngeal cancers has turned out to be related to habitual smoking, as has already been shown in lung cancers.[13,14] Thus, it is possible that p53 abnormalities in laryngeal cancers are among the characteristic genetic changes caused by the chemical carcinogens in cigarette smoke.[7,10]

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Recently, human papillomavirus (HPV) infection was verified in laryngeal cancers[11,15–18] and it has been suggested that subcellular interaction between HPV oncoproteins E6 and E7 and antioncogenes p53 and Rb might play an important role in HPV carcinogenesis in the cervix.[19–21] A possible mechanism is that HPV oncoprotein E6 binds the p53 product and promotes rapid degradation of the p53 product by involving a ubiquitin-dependent proteolytic system.[22] In cervical cancers, however, it is still controversial whether this phenomenon is actually involved in carcinogenesis.[23,24]

On these grounds, we investigated a group of laryngeal cancers in order to determine if there is a mutual relation between cigarette smoking and p53 abnormalities from the clinico-pathological and molecular biological standpoints and to determine whether p53 abnormalities including point mutation could be inversely related to HPV infection in laryngeal cancers.

MATERIALS AND METHODS

Tumors and DNA extraction Forty-one cases of laryngeal cancer were selected from the pathological files of the Gunma University Hospital during the period from 1986 to 1990. Biopsy samples obtained from the primary site of the 41 cancers had been fixed in 15% formalin and embedded in paraffin. All biopsy samples were histologi-

cally reviewed and the presence of cancer tissue was confirmed. Clinical staging of the case was determined according to the TNM classification by the UICC.

Tumor DNA was extracted from the formalin-fixed and paraffin-embedded biopsy tissues by a modified Goelz's method.[25] Briefly, several 5-μm-thick paraffin sections were dewaxed with xylene and digested with 0.01% proteinase K solution containing 1% sodium dodecyl sulfate at 48°C for 48 h, followed by repeated phenol and chloroform extraction, and the extracted DNA was precipitated with cold ethanol.

Immunohistochemical analysis for p53 Of the 41 cases with laryngeal cancer, 37 could be used for immunohistochemical analysis for p53, but cancer tissue was lost in four cases after the specimens had been subjected to DNA extraction. The paraffin section was immunostained for p53 by the avidin-biotin-peroxidase complex method after microwave treatment for 9 min at 90°C in 20% zinc sulfate aqueous solution.[26] The primary antibody (mouse monoclonal antibody against human p53; DO-7) was purchased from Dako Japan Co. Ltd., Kyoto. The reaction was continued for 3 h at room temperature. When more than 10% of tumor nuclei was stained for p53, laryngeal cancers were judged as immunohistochemically positive for p53, as previously described by Anwar *et al.*[11]

Detection and typing of HPV DNA The methods of detection and typing of HPV DNA were described in our previous paper.[18] Briefly, a fragment of about 250 base-pairs of the common L1 region of various HPV DNAs was amplified by the polymerase chain reaction (PCR) using the pair of consensus primers originally described by Yoshikawa *et al.*[27] This PCR system can amplify the common L1 region of various HPV subtypes (HPV types 6, 11, 16, 18, 31, 33, 42, 52, and 58), which are commonly harbored in the cervix and genitalia, and can detect 0.1 pg of DNA of various HPV types. After the PCR, the newly amplified DNA band was digested with several restriction enzymes (*Rsa* I, *Dde* I and *Hae* III) and the HPV subtype was determined on the basis of the restriction fragment length polymorphism (RFLP).

Detection of p53 point mutation by PCR-SSCP analysis The PCR-single strand conformation polymorphism (SSCP) analysis for the p53 gene was carried out according to the methodology described by Orita *et al.*, with a slight modification.[28] The oligonucleotide primers amplifying each exon from 5 to 8 of the p53 gene were synthesized with a DNA synthesizer (Applied Biosystems Inc., Foster City, CA) as described by Miller *et al.*[29] The 5′ end of each primer was labeled with [γ-^{32}P]ATP (6000 Ci/mmol, Amersham Japan Ltd., Tokyo) by bacteriophage T4 polynucleotide kinase (Takara Shuzo Co., Ltd., Kyoto). The PCR was carried out in a DNA Thermal Cycler (GeneAmp PCR System 9600, Perkin-Elmer Cetus, Norwalk, CT) for 35–45 cycles (denaturation for 1 min at 94°C, annealing for 1 min at 55°C and extension for 2 min at 72°C). DNAs extracted from cell lines containing a mutated p53 gene (small cell lung cancers: Lu130, Lu135 and Lu139; and a uterine cancer: EM54) and from a normal foreskin were used as controls for p53 point mutation. After the PCR, 1 μl of reaction mixture was added to 9 μl of denaturation solution (95% formamide, 20 m*M* EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), and the mixture was heated for 10 min at 95°C then rapidly cooled on ice. Then the solution was electrophoresed on 12.5% or 20% acrylamide gel (100×100×1 mm) with or without 5% glycerol for 2.5 to 5 h at 4°C or 20°C. After the electrophoresis, the gel was dried and autoradiographed on X-ray films (Kodak X-AR, Eastman Kodak Co., Rochester, NY) with intensifying screens for 1 h at room temperature.

DNA sequencing of p53 gene For DNA sequencing, a single mutated band or a pair of them was excised from the dried gel and the DNA was extracted from the gel with 30 μl of distilled water at 80°C for 15 min. Using 1 μl of extracted DNA solution as a template, the mutated DNA was purified on a membrane column (Millipore UFC3TK) or with glassmilk (Genclean, Funakoshi, Tokyo). For DNA sequencing, a dsDNA Cycle Sequencing System kit (GIBCO BRL, Grand Island, NY) was used according to the manufacturer's instruction manual with the sense or anti-sense primer endolabeled with [γ-^{32}P]ATP by bacteriophage polynucleotide kinase.

RESULTS

Immunohistochemical and molecular analysis for p53 Of the 37 laryngeal cancer specimens analyzed, 23 were immunohistochemically positive for p53. Diffuse immunoreactivity for p53 was seen exclusively in the nuclei of cancer cells (Fig. 1).

PCR-SSCP analysis detected mutated allele bands with a different mobility shift from normal allele bands in 12 (29.2%) of 41 laryngeal cancer DNAs (Fig. 2). Direct sequencing analysis was performed on these 12 cancer DNAs and revealed that all the DNAs contained a point mutation in exon 5 to 8 of the p53 gene, as summarized in Table I. When direct sequencing analysis was carried out on five cancers which were immunohistochemically p53-positive and contained HPV-DNA, and in which no mutated band was detected by the PCR-SSCP method, no base substitution from exon 5 to 8 of p53 gene was observed (data not shown). Point mutation of p53 in our cases ranged from codons 157 to 278, and point mutation in codon 157 was the most common (four cancers), followed by codon 248 (three cancers) (Fig. 3). Nucleotide substitutions in codons occurred in the first base in eight cancers, in the second base in two and in the third

base in two. G-to-C and G-to-T transversions were observed in five and two cancers, respectively, of which three were detected at CpG sites. G:C to A:T transitions were seen in five cancers, but no A:T to G:C transitions were found. All except two base substitutions were missense mutations (Table I).

p53 point mutation was detected in only 11 of the 23 immunohistochemically p53-positive cancers. Conversely, in one cancer with a point mutation in the p53 gene, p53 was not detected immunohistochemically (Table II).

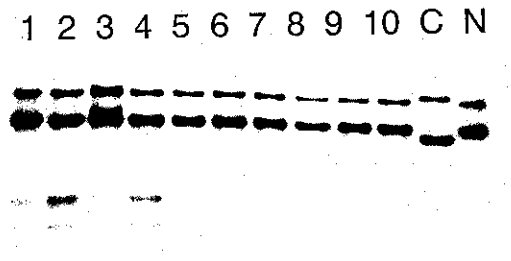

Fig. 2. PCR-SSCP Analysis of the p53 gene (exon 7) in laryngeal cancers. An additional mutated band with a mobility shift is clearly seen in case 3. C: Positive control; DNA extracted from Lu135. N: Negative control; DNA extracted from human foreskin.

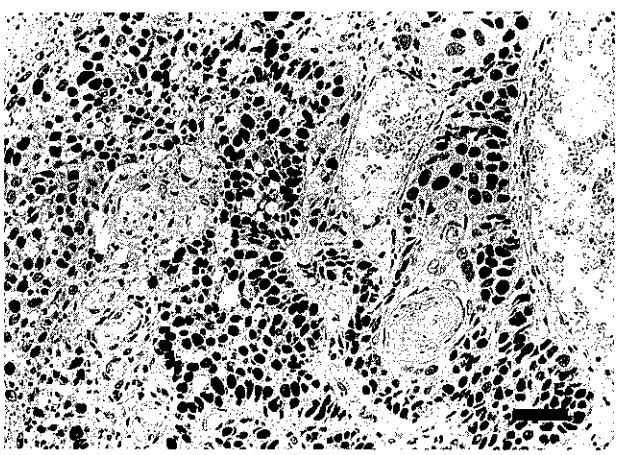

Fig. 1. Immunohistochemical demonstration of p53 in laryngeal cancers. Strong and diffuse immunoreaction for p53 is exclusively located in the nuclei of the cancer cells. Lightly counterstained with hematoxylin. Scale bar: 50 μm.

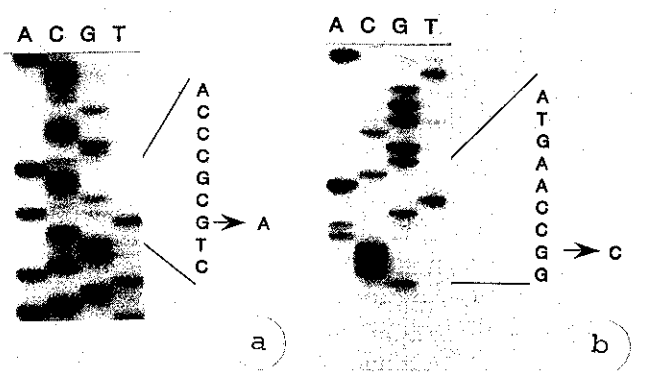

Fig. 3. Direct sequence analysis of the p53 gene in laryngeal cancers. Point mutations of the p53 gene are present in codon 157 (a) and in codon 248 (b).

Table I. p53 Mutations in Laryngeal Carcinomas

Case	Sex[a)]	Age	Location[b)]	Exon	Codon	Mutation	Immunostain[c)]	HPV
1	M	61	G	5	157	GTC(Val) → CTC(Leu)	+	–
2	M	59	G	5	157	GTC(Val) → TTC(Phe)	+	–
3	M	57	G	5	157	GTC(Val) → ATC(Ile)	–	–
4	M	62	SUP	5	157	GTC(Val) → ATC(Ile)	+	–
5	M	72	G	6	198	GAA(Glu) → CAA(Gln)	+	–
6	M	70	SUP	7	245	GGC(Gly) → TGC(Cys)	+	16
7	M	44	G	7	248	CGG(Arg) → CGC(Arg)	+	16
8	M	56	SUB	7	248	CGG(Arg) → CGA(Arg)	+	–
9	F	72	SUP	7	248	CGG(Arg) → CCG(Pro)	+	–
10	M	47	G	7	252	CTC(Leu) → TTC(Phe)	+	16
11	M	77	SUP	8	273	CGT(Arg) → CCT(Pro)	+	–
12	F	46	G	8	278	CCT(Pro) → TCT(Ser)	+	16

a) Sex: male (M), female (F).
b) Location of primary site: supraglottic (SUP), glottic (G) and subglottic (SUB).
c) Immunohistochemistry for p53: positive (+) or negative (–).

Table II. p53 Abnormalities and Presence of HPV DNA in Laryngeal Cancers

Immunohisto-chemistry for p53	Positive 23 cases		Negative 14 cases	
p53 mutation	positive 11 cases	negative 12 cases	positive 1 case	negative 13 cases
HPV-DNA-positive	4 cases	5 cases	0 case	2 cases

Table III. Correlation between Brinkman Index, p53 Abnormalities and Presence of HPV DNA in Laryngeal Cancers

	Brinkman index of smoking history	
	more than 1000 19 cases	less than 1000 22 cases
Immunohistochemically positive for p53	13 cases	9 cases
p53 mutation-positive[a)]	9 cases	3 cases
HPV-DNA-positive	5 cases	6 cases

a) Statistically significant: $P<0.05$ by two-tailed Student's *t* test.

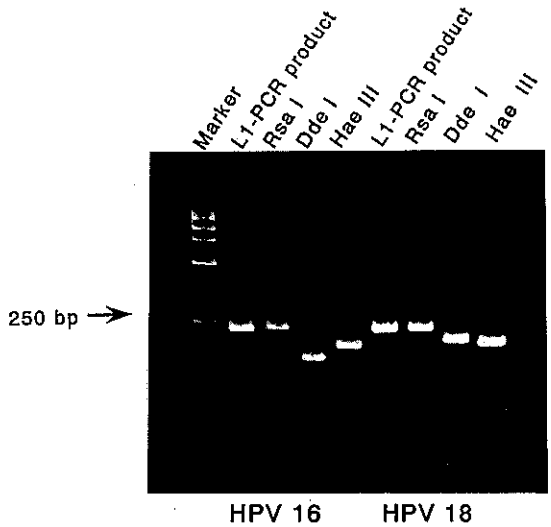

Fig. 4. Demonstration of HPV subtypes 16 and 18 by RFLP after PCR. Amplified L1 region of HPV 16 shows a different RFLP pattern from that of HPV 18. Marker: $\phi\chi$ 174-*Hae* III digest.

Clinicopathological features and p53 abnormality The 41 laryngeal cancer tissues used in this study were collected from 41 patients (36 males and 5 females) with a mean age of 62.8 years (ranging from 44 to 90 years). All the patients except one female had a history of habitual smoking (Brinkman index[30)] from 320 to 2400; average 882). Concerning the interrelation between habitual smoking and p53 point mutation, point mutation was more frequently detected in heavy smokers with an average Brinkman index score of more than 1000 than in smokers with an index below 1000 (Table III). The exception is the female non-smoker who showed point mutation of p53. As regards other clinicopathological features such as site and stage of the laryngeal cancer, there was no relation to p53 abnormality (data not shown).

HPV infection and p53 abnormality in laryngeal cancers By use of the PCR reaction for HPV DNA detection, a newly amplified specific band (about 250 bp in size) was detected in 11 (26.8%) of the 41 laryngeal cancers investigated. According to HPV subtype analysis using the RFLP pattern of the newly amplified HPV DNA, nine DNAs were HPV type 16 and the other two were HPV type 18 DNA (Fig. 4). HPV type 16 DNA was present in both glottic and supraglottic cancers, but HPV type 18 DNA was present only in two supraglottic cancers. None of the other HPV subtypes (HPV types 6, 11, 31, 33, 42, 52 and 58) was detected in this study.

HPV DNA was more frequently observed in cancers with a p53 abnormality, including immunohistochemical positivity for p53, or a p53 point mutation than in cancers without any p53 abnormality, but the difference was not statistically significant (Tables I and II).

DISCUSSION

The p53 gene mutation is the most common cancer-related genetic change at the gene level and the majority of base substitutions of p53 in human cancers lie in the region from codon 110 to 307 (from exons 5 to 8).[5,6)] With respect to the position of frequent base substitutions, at least three mutation hot spots (codons 175, 248 and 273) are present in human cancers.[5)] Moreover, p53 mutations including both transitions and transversions more frequently occur at G:C sites than at A:T sites in various human cancers.[6)]

In laryngeal cancers, nucleotide sequence analysis for p53 has been performed in at least two studies, in which abnormalities of the p53 gene including frequent G-to-T transversions and G-to-A transitions were found.[9,10)] The point mutation at codon 157 has been found only in a few cancers such as lung,[29,31)] esophagus,[32)] breast[33)] and liver cancers.[34)] This is the first report that a mutation at codon 157 is frequently present in laryngeal cancers; this may be a specific genetic change for laryngeal cancer, analogously with the frequent base substitution at codon 249 in hepatocellular carcinoma.[35,36)]

A large volume of epidemiological data has shown that cigarette smoking is the main etiological factor of laryngeal cancers, suggesting that chemical carcinogens play an important role in the carcinogenic process.[37, 38] In chemical carcinogenesis, it is well known that guanine is the preferential target for carcinogen-induced mutations and that the major tobacco-associated mutagens, benzo[*a*]pyrene and nitrosoamines, preferentially induce G-to-T and G-to-A substitutions, respectively.[39, 40] In fact, G-to-T transversions have been reported to predominate in non-small cell lung cancers associated with smoking.[13, 29] Our study shows that the majority of mutations occurred on the guanine nucleotide of the codon and that the types of base mutations were both transitions (G to A) and transversions (G to T and G to C). These results indicate that exposure to various mutagens including smoke-related carcinogens might induce laryngeal carcinogenesis.

A close relationship between p53 abnormality and laryngeal carcinogenesis is supported by previous immunohistochemical studies.[7–12] In laryngeal cancers, p53 is immunohistochemically demonstrable at a high frequency, from 60% to 73.3%.[8, 9, 11] Furthermore, p53 over-expression seems to be related not only to laryngeal carcinogenesis, but also to habitual smoking, but unrelated to the biological aggressiveness of laryngeal cancers.[7, 8, 10–12] Our immunohistochemical results for p53 corroborate previous findings that p53 abnormality may be caused by habitual smoking.

Previous immunohistochemical studies on p53 in human cancers, including laryngeal cancers, have shown that immunohistochemically demonstrable p53 is correlated with mutation of the p53 gene.[9, 10, 24, 32] Our results may suggest that nuclear accumulation of p53 in human cancers is due not only to increased stability of p53 as a result of point mutation, but also to other mechanisms which include altered expression of the wild-type p53 gene by cellular transcriptional regulators, destruction of the ubiquitin system for degradation of p53 and stabilization of p53 by formation of a complex with cellular or DNA virus protein.[41] Further studies are necessary to establish which mechanisms are involved in this phenomenon.

Recently, HPV infection, especially with HPV types 16 and 18, has been linked with the development of laryngeal cancers as well as cervical cancers.[11, 15–18] HPV is a potent tumor virus encoding oncoproteins E6 and E7, both of which inactivate the normal function of tumor-suppressor genes.[19, 20] In cervical cancers, p53 seems to be inactivated either by complexing with HPV E6 protein or by p53 gene mutation, resulting in an inverse correlation between HPV infection and somatic mutation of the p53 gene.[21] Our study showed that the p53 mutation was present in only four of 11 HPV DNA-positive laryngeal cancers. Furthermore, we could not find any evidence that p53 mutation might be closely related to the progression of HPV DNA-positive laryngeal cancers. Although HPV E6 oncoprotein closely reflects the p53 expression level in human cancers, further studies are required to resolve the role of HPV infection in laryngeal carcinogenesis.

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