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Archival Analysis of p53 Protein Overexpression and Genetic Mutation in Esophageal Squamous Cell Carcinoma

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Summary. **Alterations in p53 were analyzed by immunochemistry in 131 lesions from 108 patients and by polymerase chain reaction single-strand conformation polymorphism(PCR-SSCP) in 69 tumors from 69 patients with esophageal squamous cell carcinomas, to elucidate the correlation between protein overexpression and mutation, and between p53 alteration and clinicopathological factors. The p53 gene-product was overexpressed in 83 (63%) of the 131, and mutations were detected in 24 (35%) of the 69 tumors examined. The mutations were predominantly located at exons 5 (58%) and 7 (27%), and detected in 13 (31%) of 42 overexpressing tumors, in none of 4 sporadic tumors and in 8 (40%) of 20 negative tumors. The p53 alteration did not correlate with the clinicopathological factors, including pTNM categories, stage, depth of invasion, and size of intramucosal carcinomas. These results indicate that p53 protein overexpression and gene mutation may occur during an early stage of esophageal carcinogenesis and have no impact on clinicopathological factors. The higher frequency of p53 overexpression compared to mutation suggests that p53 accumulation can occur by other mechanisms besides p53 mutation.**

Key words—p53 overexpression, p53 gene mutation, esophageal carcinoma, clinicopathological factors.

INTRODUCTION

Alteration of the p53 tumor supressor gene reportedly plays an important role in the development and progression of human malignant tumors, is the most frequent genetic change in human cancers.[1] Alterations in p53 have been investigated by means of immunohistochemistry and DNA analysis. The former is relatively rapid and simple, and can demonstrate correlations between p53 alterations and various histopathological findings. In esophageal squamous cell carcinomas, Wang et al.[2] have found that the accumulation of p53 protein is related to invasiveness and metastatic potential. However, Shimaya et al.[3] and Sarbia et al.[4] did not find any correlation between p53 expression and clinicopathological factors.

It has been reported that p53 protein accumulation corresponds to p53 missensse mutations.[5-7] However, p53 mutations are not necessarily associated with p53 protein overexpression, and may occur without mutations in exons 5-8.[8,9]

To gain insight into the role of p53 gene in esophageal squamous cell carcinomas in this study, we investigated the correlation between p53 alteration and clinicopathologic parameters in 131 carcinomas by means of immunohistochemistry as well as the correlation between p53 protein overexpression and p53 mutation in 69 carcinomas by means of the polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) methods.[10]

MATERIALS AND METHODS

Materials

Surgical specimens from 131 primary esophageal squamous cell carcinomas (108 patients) were obtained from the archives of the First Department of Pathology at Niigata University from August, 1988 to July, 1994. There were 94 males (40 to 85 years of age with a mean ± SD of 64.4 ± 8.3 years)

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and 14 females (50 to 76 years of age with a mean ± SD of 64.6 ± 8.3 years). None of the patients had received any radiation therapy or chemothrapy before surgery. All resected specimens were fixed in 10% formalin. The tumor size and depth of invasion were determined by iodine-staining and by examining 3 μm hematoxylin-eosin (HE) sections from 5×30 mm, consecutive, serially paraffin-embedded blocks of the entire resected specimens. The histopathological findings were mapped on color prints. Histological diagnoses were made according to the modified criteria[11] established by the World Health Organization.[12] Pathological staging was based on the TNM classification.[13] We studied the size and histological grading of tumors, tumor depth (pT) and lymph node status (pN). Distant metastasis (M) was defined by preoperative clinical data.

Immunohistochemical analysis of p53 protein

Three serial, 3 μm-thick sections were made from representative blocks of each carcinoma. The first section was stained with HE, the second immunostained for p53 using the mouse monoclonal antibody PAb1801 (Oncogene Science Inc., Manhasset, NY, USA), and the third was immunostained for Ki-67 (MIB1; Immunotech, Marseille, France). Immunohistochemical staining was performed using the streptavidin-peroxidase complex.[14] Eleven (8%) of the 142 tumors initially selected for this study did not show Ki-67 positivity for the inner control such as nonnepolastic basal and parabasal cells, and germinal center cells of the lymph follicles. Therefore, these tumors were excluded from this study because the p53 positivity in these specimens was lost or markedly decreased in the tumor tissues as well as in nonneoplastic mucosa. A total of 131 tumors were analyzed for the p53 protein.

Cells positive for p53 were defined as those with a brown-stained nucleus, regardless of staining intensity. Tumors were scored as positive when at least one positive nucleus was visible within the lesion. Three p53 staining patterns were idntified; 1) diffuse, with many positive cells distributed throughout most of the lesion; 2) nested where positive cells aggregated in focal area(s); and 3) scattered, having a few isolated positive cells scattered in the lesion.[14] The overexpression of p53 was defined as nested or diffuse according to our published studies.[11,14,15]

DNA preparation

Tissue sections from ten serial, 10 μm-thick sections of pTis and pT1 carcinomas and five serial, 10 μm-thick sections of pT2/3 carcinomas were dewaxed for 5 min in two changes of xylene and rehydrated for 5 min in two changes of alcohol. For pTis and pT1 carcinomas, the entire regions containing the cancer cells were dissected under a microscope, for pT2/3 carcinomas, they were dissected macroscopically. The samples were placed in a 1500 μl Eppendorf tube with 200 μl HMW buffer, and then 10 μl of 10% SDS and proteinase K were added to a final concentration of 200 μg/ml to each tube and incubated for 12 h at 48°C. The mixture was successively extracted with equal volumes of TE buffer-saturated phenol (pH 8.0): chloroform: isoamylalcohol (25 : 24 : 1 v/v/v) until the protein interface became invisible. DNA was precipitated with ethanol and the DNA pellet was dried and dissolved in 20 μl of sterile water.

The first primers		
	Exon 5	5′-AACTCTGTCTCCTTCCTCTT-3′
		5′-AACCAGCCCTGTCGTCTCTC-3′
	Exon 6	5′-GCCTCTGATTCCTCACTGAT-3′
		5′-TTAACCCCTCCTCCCAGAGA-3′
	Exon 7	5′-AGGTCTCCCCAAGGCGCACT-3′
		5′-CAGGGTGGCAAGTGGCTCCT-3′
	Exon 8	5′-TTCCTTACTGCCTCTTGCTT-3′
		5′-AGGCATAACTGCACCCTTGG-3′
The second primers		
	Exon 5	5′-GTCTCCTTCCTCTTCCTACA-3′
		5′-TGTCGTCTCTCCAGCCCCAG-3′
	Exon 6	5′-TCTGATTCCTCACTGATTGC-3′
		5′-TCCTCCCAGAGACCCCAGTT-3′
	Exon 7	5′-AAGGCGCACTGGCCTCATCT-3′
		5′-GGTGGCAAGTGGCTCCTGAC-3′
	Exon 8	5′-CTTACTGCCTCTTGCTTCTC-3′
		5′-CATAACTGCACCCTTGGTCT-3′

Fig. 1. PCR amplification primers.

Polymerase chain reaction

Oligonucleotide primers were synthesized by Takara Shuzo Co., Ltd and Funakoshi Co., Ltd. Four fragments of DNA, including exons 5, 6, 7, and 8 of the p53 gene were amplified by nested PCR[16] using two sets of primers for each exon. The first and second PCR amplification primers are shown in Fig. 1. The first PCR reactions proceeded in a 50 μl reaction mix containing 50 mM potassium chloride, 10 mM TRIS-HCl (pH 8.0), 0.1% Triton X-100, 1.5 mM magnesium chloride, 200 μM of each nucleotide (dATP, dCTP, dGTP, dTTP., Takara Shuzo Co., Ltd, Tokyo,

Table 1. Relationship between p53 immunoreactivity and clinicopathological factors in patients with esophageal squamous cell carcinoma

	No. of tumors examined	No. of tumors by p53 immunostaining pattern				p53 mutation (+)	
		Negative	Scattered	Nested	Diffuse		
TNM classification[a)]							
pTis	26	8 (31%)	2 (8%)	1 (4%)	15 (57%)	2/ 7 (29%)	
pT1a	25	5 (20%)	1 (4%)	0	19 (76%)	2/ 7 (29%)	
pT1b	35	12 (34%)	1 (3%)	0	22 (63%)	16/30 (53%)	
pT2	11	3 (27%)	0	0	8 (73%)	1/ 4 (25%)	$p<0.01$
pT3/4	34	16 (47%)	0	0	18 (53%)	3/21 (14%)	
pN0	58	18 (31%)	3 (5%)	1 (2%)	36 (62%)	17/40 (43%)	
pN1	50	20 (40%)	1 (2%)	0	29 (58%)	7/29 (24%)	
M0	95	34 (36%)	4 (4%)	1 (1%)	56 (59%)	24/66 (36%)	
M1	13	4 (31%)	0	0	9 (69%)	0/ 3 (0%)	
Size of tumor (mm)							
$0<x\leqq 5$	14	3 (23%)	0	1 (8%)	10 (69%)	0/ 0	
$5<x<10$	7	2 (29%)	0	0	5 (71%)	0/ 1 (0%)	
$10\leqq x<20$	12	5 (42%)	0	0	7 (58%)	2/ 5 (40%)	
$20\leqq x$	98	34 (35%)	4 (4%)	0	60 (61%)	22/63 (34%)	
Histological grading[b)]							
G1	25	12 (48%)	1 (4%)	1 (4%)	11 (44%)	3/14 (21%)	
G2	90	28 (31%)	3 (3%)	0	59 (66%)	16/44 (36%)	
G3	16	4 (25%)	0	0	12 (75%)	5/11 (45%)	
Stage of carcinoma							
0	13	6 (46%)	1 (8%)	1 (8%)	5 (48%)	2/ 6 (33%)	
I	34	9 (27%)	2 (6%)	0	23 (67%)	12/25 (48%)	
IIA	10	3 (30%)	0	0	7 (70%)	3/ 8 (38%)	
IIB	18	6 (33%)	1 (6%)	0	11 (61%)	6/13 (46%)	
III	19	10 (53%)	0	0	9 (47%)	1/13 (8%)	
IV	14	4 (29%)	0	0	10 (71%)	0/ 4 (100%)	

[a)]; pTis: carcinoma *in situ*, pT1a: tumor invades lamina propria, pT1b: tumor invades submucosa, pT2: tumor invades muscularis propria, pT3: tumor invades adventitia, pT4: tumor invades adjacent structures, pN0: no regional lymph node metastasis, pN1: regional lymph node metastasis, M0: no distant metastasis, M1: distant metastasis.
[b)]; G1: well differentiated squamous cell carcinoma, G2: moderately differentiated squamous cell carcinoma, G3: poorly differentiated squamous cell carcinoma.

Japan), 20 pmole of each primer, 2.5 units of Taq DNA polymerase (Promega Corp.), and 1 or 3 μl of DNA template. After one cycle of 94°C for 5 min, 55°C for 1 min 30 sec, 72°C for 1 min, 35 cycles of 94°C for 1 min, 55°C for 1 min 30 sec, or 72°C for 1 min had proceeded, a 7 min extension at 72°C using the Program Temp Control System PC-700 (ASTEC Co., Ltd., Fukuoka, Japan) followed. The product of the first PCR was used as the template for a second PCR. Second PCR rections were performed under the same conditions as the first, except that annealing was performed at 60°C. In each experiment, two control reactions, containing no template DNA (Blunk) or Human placental DNA (Oncogene Science), were always performed simultaneously with sample reactions. The products of the second PCR were precipitated with ethanol and purified from 4% agarose gels (NuSieve 3 : 1 Agarose: FMC BioProducts) using the Mermaid Kit from Bio101 (La Jolla, CA, USA). Informative cases were defined as those with amplified bands on the agarose gels.

SSCP analysis

The PCR products of exons 5, 6, 7, and 8 of the p53 gene were labeled at the 5′ ends with [γ-^{32}P] ATP.

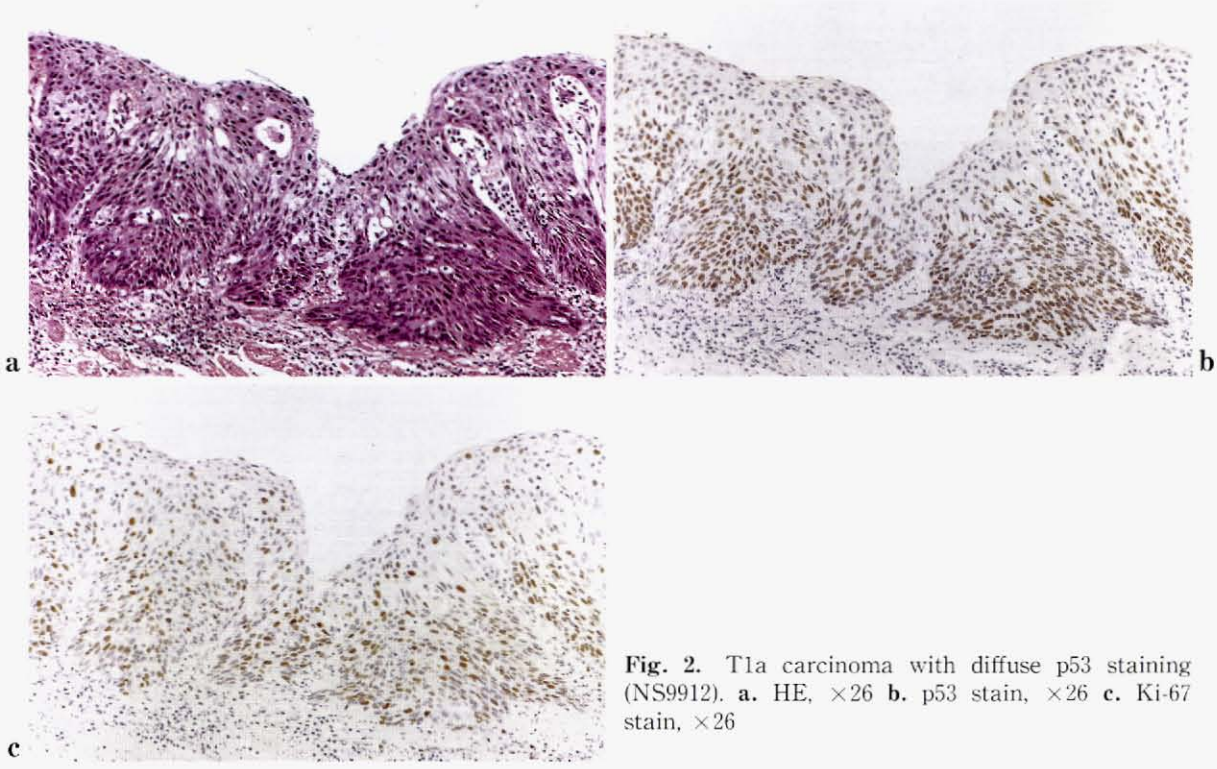

Fig. 2. T1a carcinoma with diffuse p53 staining (NS9912). **a.** HE, ×26 **b.** p53 stain, ×26 **c.** Ki-67 stain, ×26

Table 2. Frequency of p53 mutation in esophageal squamous cell carcinoma

Primary tumor	No. of tumor (area*; mm²)	No. of not informative tumor (area*; mm²)	No. of informative tumor (area*; mm²)	No. of mutation	
Tis	12 (3.0± 3.4)	5 (1.3± 1.4)	7 (4.2± 3.9)	2 (29%)	
T1a	11 (6.1± 4.3)	4 (6.2± 4.6)	7 (6.1± 4.5)	2 (29%)	
T1b	39 (14.8±14.7)	9 (21.2±16.9)	30 (13.4±13.9)	16 (53%)	p<0.01
T2/3	25 (166.2±76.3)	0	25 (166.2±76.3)	4 (16%)	
Total	87	18	69	24 (35%)	

Tis: Carcinoma *in situ*, T1a: Tumor invades lamina propria (mucosa), T1b: Tumor invades submucosa, T2: Tumor invades muscularis propria, T3: Tumor invades adventitia.
*: Size of area dissected from a paraffin-embedded block for p53 mutation analysis.

The labeled DNA was precipitated with ethanol and the DNA pellet was dried and dissolved in 15 μl of a loading solution containing 95% deionized formamide, 10 mM EDTA and 0.05% bromophenol blue and xylene cyanol. After denaturation at 94°C for 3 minutes, 1–2 μl of the mixture was resolved on a 6% polyacrylamide gel (29 : 1 acrylamide: bisacrylamide ratio) containing 0.6×TBE buffer and 5% glycerol. The gel was run at 30 W for 4–5 h and cooled at 25°C using a MultiTemp II (Pharmacia Biotech). The gel was then dried and exposed to X-ray film (Fuji).

Statistics

Differences in the frequency of p53-positive samples were evaluated by the Chi-squared test, and differences in the p53 staining pattern by Fisher's exact test. Probability values less than 0.01 were considered statistically significant.

RESULTS

The overexpression of p53 protein was detected in 83 (63%) of 131 informative tumors. In one of them, it

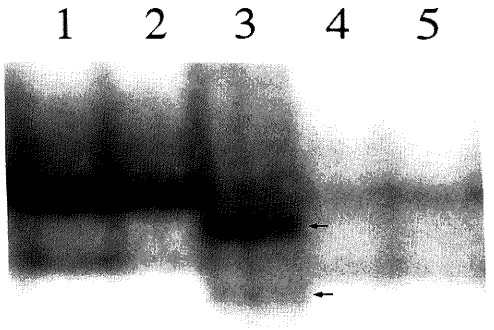

Fig. 3. Representative SSCP analysis of p53 gene exon 7 in esophageal squamous cell carcinomas. Lane 1, negative control (human placenta); Lane 3, T1b carcinoma with p53 mutation (NS10704); Lane 2, 4 and 5 carcinoma without p53 mutation (NS10695, 10709, 10772). Bands showing mobility changes. (*arrows*)

Table 3. Relationship between p53 immunoreactivity and mutation in esophageal squamous cell carcinoma

p53 staining pattern	No. of informative tumors	Mutation (+)	Mutation (−)
Negative	20	8 (40%)	12 (60%)
Scattered	4	0	4 (100%)
Diffuse	42	13 (31%)	29 (69%)
Total	66	21 (32%)	45 (68%)

was expressed in the manner of a nested pattern, whereas it was diffuse in the others, regardless of the depth of cancerinvasion (Table 1 and Fig. 2). The p53 immunoreactivity was scattered in 4 of 131 tumors and often appeared in the same pattern in non-neoplastic normal mucosa. The p53 immunostaining did not correlate with pT, pN, or M categories, size, histological grade or stage of carcinoma ($p>0.01$) as shown in Table 1. Even in pTis and pT1a carcinomas, the overexpression rate was similar among tumors regardless of size.

Genetic DNA was purified from 87 tumors (pTis/pT1: 62, pT2/pT3; 25). The area of samples did not differ between the informative and non informative tumors of each pT category ($p>0.01$). Overall, 69 of 87 tumors were informative by PCR and were further analyzed by SSCP (Table 2). The p53 mutation was detected in 24 of 69 (35%) of the informative tumors. Regardless of the significantly smaller area of samples examined (Table 2) and the similar ratio of the semiquantitatively determined cancer tissue in the samples (60-90%, average 80% in T1b, and 40-90%, average 70% in T2/3), pT1b carcinomas had significantly more mutations than pT2 and pT3 carcinomas (16/30 vs 4/25, $p<0.01$). The p53 mutations also did not correlate with pN or M categories, size, histological grade or stage of carcinoma ($p>0.01$) as shown in Table 1.

Of the 24 tumors with mutations, 15 (56%) had the mutation in exon 5, 7 (26%) in exon 7 (Fig. 3), 2 (8%) in exon 6, 2 (8%) in exon 8, one in exon 5 and 6, and one in exon 5 and 7.

This study did not reveal any correlation between p53 immunoreactivity and p53 mutation (Table 3).

DISCUSSION

Of 131 squamous cell carcinomas we found p53 immunoreactivity and overexpression in 87 (66%) and 83 (63%), respectively. This frequency was consistent with the results of others on esophageal squamous cell carcinomas,[3,17,18] where the p53 immunoreactivity of tumors was in the range of 53 and 80%.

Some of these publications refer to p53 positive cell accumulation or p53 immunoreactivity as a synonym for p53 protein overexpression in esophageal cancers, because of the absence of p53 positive cells in the non-neoplastic or normal mucosa. However, p53 positive cells have been found scattered in non-neoplastic mucosa by some researchers[2] as well as in this study. Therefore, we defined p53 overexpression as the nested or diffuse presence of p53 immunoreactive cells. Watanabe et al.[11] have found that the p53 labeling index of esophagitis increased more during the regenerative stage but was lower than that of esophageal carcinoma, and always lower than the Ki-67 labeling index in the corresponding areas, contrary to a higher p53 labeling index in carcinomas. They concluded that this finding might be useful for diagnosing malignant transformation. The focal and diffuse p53 immunoreactivity found in this study corresponded more to a group with higher p53, than with the Ki-67 labeling index. A clear definition of p53 overexpression is important when describing the correlation of p53 alterations with clinicopathological factors and p53 mutations.

It is controversial whether or not p53 overexpression is correlated with clinicophathologicgal factors. Wang et al.[2] have found that p53 protein overexpression was related to the invasiveness and metastatic potential of cancer cells. However, other investigators[3,4] did not find any correlation. This difference might be

due to methodology, the antibody, and the definition of p53 protein overexpression. In this study, neither p53 overexpression nor p53 mutation correlated with pT, pN, or M categories, size, histological grade of tumor or TNM staging (Table 1). The significantly lower incidence of p53 mutations in pT3 than in pT1b carcinomas may be due to the selection of materials, because the semiquantitative determination of cancer tissue in samples was similar (60-90%, average 80%, in pT1b, and 40-90%, average 70%, in T2/3). It therefore is necessary to analyse more samples to explain this difference.

We detected p53 mutations in 24 (35%) of 69 informative tumors. This frequency was lower than the 84% in esophageal carcinomas in western France[19] reported by Audrezets' et al. ($p<0.01$), but consistent with the 38-55% recently reported for esophageal carcinomas in China[20,21] and Japan.[22,23] This difference in frequency may result from the methodology and materials. For example, Audrezets' et al. used a more sensitive method, i.e., GC clamp denaturing gradient gel electrophoresis.[19]

In p53 mutations of human esophageal cancers, exons 5, 6, 7, and 8 are considered to be equally affected.[21,24] However, in Henan, China,[20] exons 5 (59%) and 7 (29%), and in Brittany, France,[19] exon 6 (41%) were predominantly mutated. In this study, 58% of the mutations were found in exon 5 and 27% in exon 7. These results are very similar to those from Henan, China, where the causative agents are thought to be O^6-methyl-thymine and N-Nitroso compounds.

A correlation between p53 immunoreactivity and mutation on esophageal cancer was found in previous reports,[20,25,26] but not here. Our material may have been biased for p53 overexpression tumors without mutation, so that the accumulation of p53 protein may be due to a p53 mutation-independent mechanism, such as binding to molecules of cellular or viral origin, and/or the p53 mutations may occur in exons other than 5, 6, 7, and 8.

From these data, we conclude that p53 alterations may occur during an early stage of esophageal carcinogenesis and be associated with the transition from benign to malignant, without a correlation with clinicopathological factors.

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