—Original—

Detection of Circulating Anti-p 53 Antibodies in Esophageal Cancer Patients

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Abstract

It has been reported that circulating anti-p53 antibodies (p53-Ab) in the serum are detected in some cancers. To investigate the usefulness of detecting p53-Ab, we measured the circulating p53-Ab in comparison with squamous cell carcinoma antigen (SCC-Ag) in patients with esophageal carcinoma. Serum specimens from 46 esophageal cancer patients (42 squamous cell carcinomas, 3 mucoepidermoid carcinomas and 1 basaloid squamous carcinoma) and 13 healthy subjects were studied. Serum p53-Ab was measured by an enzyme-linked immunosorbent assay. Surgically resected specimens from 43 patients were immunohistochemically stained for p53. Serum SCC-Ag was measured by a radioimmunoassay. The results were analyzed with the clinical data and outcome. Serum p53-Ab was detected in 13 (28%) of the 46 patients, but not in any of the healthy subjects. The positive rate was 0% (0/6) in stageI, 60% (3/5) in stage IIA, 30% (3/10) in stage IIB, 29% (7/24) in stage III and 0% (0/1) in stage IV. There was no difference in the outcome between the p53-Ab-positive and p53-Ab-negative patients. Immunohistochemically, 30 (70 %) of the 43 specimens stained positively for p53. Serum p53-Ab was detected in 43% (13/30) of the patients with tumors which stained positively for p53. There was a close correlation between positivity for p53 immunostaining and positivity for p53-Ab ($p<0.01$). An elevated level of SCC-Ag was found in only 13% of the patients, and most patients positive for SCC-Ag already had advanced disease with lymph node metastasis and invasion to the adventitia. In conclusion, serum p53-Ab was detected in Japanese esophageal cancer patients at a frequency similar to that reported in Western countries. Serum p53-Ab may be a potentially useful molecular marker for detection and screening of esophageal cancer. Further studies of a large population may be required to elucidate the true diagnostic usefulness of measuring the serum p53-Ab. (J Nippon Med Sch 2000; 67: 110—117)

Key words: serum anti-p53 antibody, p53, esophagus, carcinoma, tumor marker

Introduction

Esophageal carcinoma is the fifth most common carcinoma in Japan. Its incidence has been increasing even in Western countries[1]. The prognosis of this disease is unfavorable in spite of advances in therapies[2]. Most carcinomas of the esophagus are already advanced at diagnosis, and therefore, detection at an early stage is crucial. Serum squamous cell carcinoma antigen (SCC-Ag) is being used as a tumor marker for esophageal carcinoma, but its sensitivity and specificity are low[3,4]. Therefore, other useful clinical markers are hoped for.

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Mutations of the p53 gene are common gene alterations in most malignant tumors, including esophageal carcinoma[5-9]. Mutant p53 protein is accumulated in the cell because of its longer half life compared with the wild-type protein[10]. Therefore, p53 overexpression can be detected by immunohistochemical staining for p53. It is reported that p53 gene alterations and/or accumulation are related to the poor response to therapy and prognosis of patients with carcinomas of the colon, breast, lung and esophagus[11-19]. Since p53 mutations are detected even in precancerous lesions, they are thought to be related to carcinogenesis[20,21]. Recently, circulating anti-p53 antibodies (p53-Ab) have been detected in the sera of patients with various carcinomas. It was reported that the presence of p53-Ab correlates closely with p53 overexpression and/or mutation[22,23]. Furthermore, p53-Ab was observed in the sera of high-risk subjects before clinical diagnosis[24,25]. However, the clinical relevance of p53-Ab, such as its usefulness for predicting the outcome or recurrence, has not been fully studied[22-32].

To clarify whether p53-Ab is useful as a clinical marker for esophageal carcinoma, we investigated the relationship between p53-Ab and clinicopathologic factors, survival and p53 immunohistochemistry. Further, the prevalence of serum p53-Ab was also compared with SCC-Ag in patients with esophageal carcinoma.

Materials and Methods

(1) *Patient characteristics*

A total of 46 esophageal cancer patients consisting of 39 males and seven females were retrospectively studied. The mean age was 62 years (range 48～78). According to the UICC classification, six patients were in stage I, five in stage IIA, 10 in stage IIB, 24 in stage III and one in stage IV. Forty-three patients underwent esophagectomy with lymph node dissection. Histologically, squamous cell carcinomas were classified as poorly differentiated (17 cases), moderately differentiated (19 cases) and well differentiated (6 cases). The others consisted of three cases of mucoepidermoid carcinoma and one case of basaloid squamous carcinoma (**Table 1**). The survival results were analyzed in 34 patients.

(2) *Preparation of sera and tissues*

Serum samples were collected from the 46 patients prior to treatment and from 13 healthy subjects as controls in the Department of Surgery I, Nippon Medical School Hospital, Tokyo, Japan. The 59 samples were stored at −80℃ until analysis. Surgical tissues were obtained from 43 patients immediately after resection.

(3) *ELISA assay for p53-Ab*

Detection of p53-Ab in sera was performed using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) (Dianova, Hamburg, Germany). After adding sera to the wells of a microtiter plate coated with recombinant p53 protein, peroxidase-conjugated goat anti-human IgG antibody was added. After incubation for 30 min at room temperature, the optical density of each well was measured at 450 nm. Positive control sera, containing a constant amount of p53-Ab, were obtained from Dianova. All samples were assayed in duplicate and were considered to be positive when showing an optical density above that of the low positive control samples from Dianova.

(4) *p53 immunostaining*

The 43 resected specimens of the esophageal tumors were fixed in 10% neutral formalin and embedded in paraffin blocks by conventional techniques. The entire specimen had been cut and blocked at a thickness of 5 mm, and the paraffin blocks were sliced serially into 5 μm sections. As pretreatment for p53 staining, sections were placed in 10 mM citric acid monohydrate buffer (pH 6) and boiled in a pressure pot under 2 atmospheric pressures for 2 min. Immunohistochemical staining was performed using a streptavidin-biotin staining technique (Histofine SAB-PO (M) kit, Nichirei, Tokyo, Japan). The sections were treated with 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity and then incubated with 10% normal rabbit serum to block nonspecific binding of the antibody. Next, they were incubated with anti-p 53 monoclonal antibody DO 7 (Novocastra, Newcastle, UK) diluted 1: 100, at room temperature for 2 h and then incubated with biotinylated rabbit anti-mouse IgG + IgA + IgM for 10 min. Finally, they were incubated with peroxidase streptavidin for 30 min. Between each step, the sections were washed

Table 1 Clinicopathologic factors in esophageal carcinomas with or without anti-p53 antibodies(p53-Ab) and squamous cell carcinoma antigen(SCC-Ag) in serum

Factor	p53-Ab positive/negative (n = 13/33)	p value	SCC-Ag positive/negative (n = 6/40)	p value
Age(mean)	64/60	0.26	63/61	0.51
Sex				
male	10/29	0.91	6/33	0.34
female	3/4		0/7	
Histology				
squamous cell ca				
well	2/4	0.89	0/6	0.41
moderate	5/14		4/15	
poor	4/13		2/15	
mucoepidermoid ca	1/2		0/3	
basaloid ca	1/0		0/1	
Depth of invasion *				
T1	2/11	0.39	1/12	0.34
T2	2/7		0/9	
T3	9/14		5/18	
T4	0/1		0/1	
Lymph node metastasis				
positive	10/25	0.63	6/29	0.17
negative	3/8		0/11	
TNM stage *				
Ⅰ	0/6	0.26	0/6	0.53
ⅡA	3/2		0/5	
ⅡB	3/7		1/9	
Ⅲ	7/17		5/19	
Ⅳ	0/1		0/1	

* according to the UICC classification

three times in phosphate buffer solution for 5 min. Diaminobenzidine-hydrogen peroxidase was used as a chromogen, and Mayer's hemotoxylin stain was used as a counterstain. Tissue sections were evaluated for cells expressing brown granules in their nuclei without cytoplasmic staining. Specimens in which over 10% of the cancer cells were immunostained for p53 were classified as positive.

(5) *SCC-Ag assay*

SCC-Ag in sera was tested with a commercially available radioimmunoassay kit (SCC・Riabead, Dainabot, Tokyo, Japan). After adding 50 μl of SCC antigen standard and sample sera to the test tubes, 100 μl of I^{125} anti-SCC antigen was added and mixed gently. After placing anti-SCC antigen beads into each test tube, the reaction mixture was incubated for 1.5 h at 20 to 30℃, and agitated at 200 ± 20 rpm on shaker. The tubes were washed three times with distilled water and then the radioactivity of all beads was measured. The SCC-Ag concentration in the specimens was determined from the SCC-Ag standard curve. A cut off concentration of 1.5 ng/ml was recommended by the manufacture.

(6) *Statistical analysis*

Comparison between the patients' clinical data and the detection of serum p53-Ab was performed with Fisher's exact probability test and the chi-square test for independence. Survival curves were constructed using the Kaplan-Meier method, and differences between the curves were tested using the log-rank test. A p-value of less than 0.05 was considered to be statistically significant.

Results

1. Serum p53-Ab and clinicopathologic factors

Serum p53-Ab was detected in 13 (28%) of the 46 patients with esophageal carcinoma (**Table 1**), while

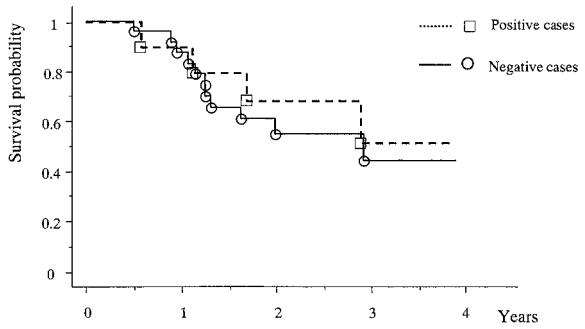

Fig. 1a Kaplan-Meier survival curves of patients with esophageal carcinoma. There is no significant difference in survival between the positive (□) and negative (○) cases for anti-p53 antibodies (p53-Ab) (p=0.66).

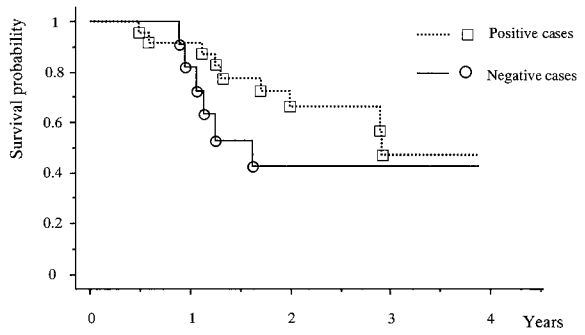

Fig. 1b Kaplan-Meier survival curves of patients with esophageal carcinoma. There is no significant difference in survival between the cases in which p53 overexpression were positive (□) or negative (○) (p=0.24).

it was not detected in any of the 13 healthy subjects. The positive rate of p53-Ab was 15% (2/13) for T1, 22% (2/9) for T2, 39% (9/23) for T3 and 0% (0/1) for T4. p53-Ab was detected in 0% (0/6) of stage I, 40% (6/15) of stage II, 29% (7/24) of stage III and 0% (0/1) of stage IV cases. The positive rate for p53-Ab did not relate to the age, gender, histological type, or differentiation of the tumor. Lymph node metastasis was also independent of the presence of serum p53-Ab (**Table 1**).

2. Serum p53-Ab and outcome

Fig. 1a shows the relationship between the overall survival curve and serum p53-Ab. There was no significant difference in the outcome between the patients who were positive and negative p53-Ab (p=0.66).

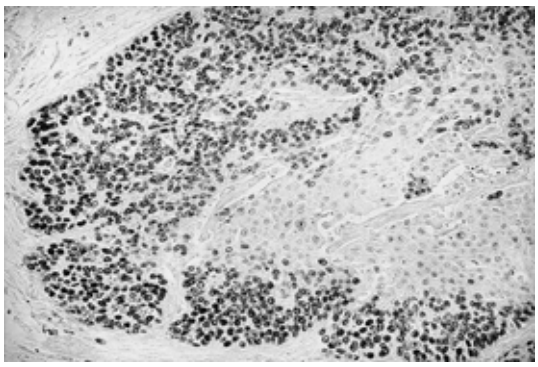

Fig. 2 Microphotograph revealing positive staining for p53 in the nuclei of carcinoma cells (×200).

Table 2 The relationship between anti-p53 antibodies (p53-Ab) and p53 immunostaining

	p53-Ab positive (n = 13)	p53-Ab negative (n = 30)	p value
p53 staining			
positive	13	17	p = 0.003
negative	0	13	

3. p53 immunostaining

p53 immunoreactivity was not found in the intact esophageal mucosa but was present in the nuclei of carcinoma cells (**Fig. 2**). p53 protein overexpression was detected in 30 (70%) of the 43 resected tumors. Serum p53-Ab was detected in 43% (13/30) of the patients with positively stained tumors. Serum p53-Ab was negative in all the patients whose tumors were negative for p53. There was a close correlation between positivity for p53 immunostaining and positivity for p53-Ab (p<0.01) (**Table 2**). However, there was no significant difference in the outcome between patients found to be positive or negative for p53 overexpression (p=0.24) (**Fig. 1 b**).

4. Serum SCC-Ag

An elevated value of SCC-Ag was found in only 6 (13%) of the 46 patients. The positive rate of SCC-Ag was 8% (1/13) for of T1, 0% (0/9) for T2, 22% (5/23) for T3 and 0% (0/1) for T4. It was detected in 0% (0/6) of stage I, 7% (1/15) of stage II, 21% (5/24) of stage III and 0% (0/1) of stage IV. The positive rate of SCC-Ag in the sera was independent of the age, gender, histological type and differentiation of the tumor. Lymph node metastasis was detected in all patients

who were positive for SCC-Ag. In all except one patient, the tumors with positive SCC-Ag invaded into the adventitia and were stage III (**Table 1**). Three patients with elevated SCC-Ag were inoperable cases because of advanced tumors which invaded the adjacent organs. A palliative operation was performed in one patient. Although it did not reach statistical significance, an elevated SCC-Ag level tended to be related to an advanced disease stage associated with lymph node metastasis and/or adventitia invasion.

Discussion

Recently, circulating p53-Ab has been reported to be detected in the serum or plasma of patients with various carcinomas. Detection is by a simple and rapid ELISA procedure. The positive rate for p53-Ab was reported to be 24% for lung cancer, 19% for pancreatic cancer and 25% for colorectal cancer[24, 33, 34]. The frequency of positive p53-Ab in patients with esophageal carcinoma ranges from 25% to 53% in the literatures[35-37].Our study detected p53-Ab in 13 (28%) of 46 patients with esophageal carcinoma. This positive rate for p53-Ab in esophageal cancer patients is thus similar to that in the published literatures. Immunohistochemically, p53 overexpression has frequently been found in esophageal carcinoma[5-8, 11-13]. It was also reported that there is a good correlation between the presence of p53-Ab in the serum and p53 overexpression in tissue samples[24, 36, 38-42]. In this study, p53 overexpression was detected in 70% of the resected esophageal tumors. Serum p53-Ab was positive in 43% of the patients with tumors which stained positively for p53. There was a close correlation between positivity for p53 immunostaining and positivity for serum p53-Ab. It is considered that accumulated p53 protein was released during cell necrosis or translocated to the surface of the cell before B-cells produce p53-Ab. However, some cases with p53 overexpression were found to be negative for p53-Ab. Stabilization and accumulation of p53 protein may be essential for antibody production, and complexes of p53 protein and 70-kDa heat-shock protein or viral protein may elicit an immune response due to altered antigen processing[22, 43, 44]. Certain conformational changes may lead to variant proteolytic cleavage of mutant p53, yielding novel peptides for MHC presentation[45]. It is possible that the production of p53-Ab is also affected by the immune status of patients[46]. The lower positive rate for p53-Ab than that of p53 staining found in this study may have been caused by modification of the above multiple mechanisms.

p53 overexpression and/or mutation of p53 protein have been demonstrated to be associated with a poor outcome in patients with carcinoma of the esophagus, lung, breast and colon[8, 16, 47]. Further, several reports found that the presence of p53-Ab relates to the outcome of carcinomas of the stomach, colon and breast[37, 42, 48]. However, it has been reported in recent years that there is no correlation between p53 overexpression and the outcome of patients with esophageal carcinoma[49,50]. In this study, no correlation was found between p53 overexpression and the outcome of the patients. p53-Ab also had no correlation with the clinicopathologic factors, including the outcome. The relationship between the presence of serum p53-Ab and the outcome thus remains controversial in esophageal cancer.

At present, among the available tumor markers, SCC-Ag is used most commonly for detection of esophageal squamous cell carcinoma. However, SCC-Ag has low sensitivity and is hardly elevated in patients with early stage disease[3, 4]. In this study, the positive rate of SCC-Ag was only 13%, and a majority of those patients already had advanced disease, with lymph node metastasis and invasion to the adventitia. Furthermore, over half of the patients who were positive for SCC-Ag could not be treated by curative operation. In the patients with T1 or T2 and stage II disease, positive rate of p53-Ab was higher than that of SCC-Ag. None of the healthy volunteers in this study were positive for p53-Ab, and few false-positive cases have been described in other reports[23, 42, 48, 51]. p53 mutation and overexpression are considered to be early events in the carcinogenesis of various carcinomas, and are seen in dysplastic epithelium of the esophagus[20, 21, 52-58]. Furthermore, in the subpopulation at high-risk of lung cancer due to exposure to vinyl chloride, p53-Ab was detected in the serum even before clinical detection of cancer[25]. These results indicate that p53-Ab can be detected in patients with stage IIA. Therefore, future studies are needed to examine the

presence of serum p53−Ab in patients with precancerous lesions such as Barrett's esophagus and the usefulness of this marker for detection of esophageal cancer in subjects at high-risk due to epidemiological factors such as smoking and alcohol consumption.

In conclusion, serum p53−Ab appears to have potential as a useful molecular maker for the detection and screening of esophageal cancer, although the relationship between genetic alterations of p53 and the clinical significance of p 53-Ab needs to be clarified.

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