Detection of $p53$ Gene Mutations in Human Esophageal Squamous Cell Carcinomas Using a $p53$ Yeast Functional Assay: Possible Difference in Esophageal Carcinogenesis Between the Young and the Elderly Group

Eiki Okuda, Harushi Osugi, Keiichiro Morimura, Nobuyasu Takada, Masashi Takemura, Shoji Fukushima, Masayuki Higashino, and Hiroaki Kinoshita
Second Department of Surgery [E. O., H. O., N. T., M. T., H. K.] and Department of Pathology [K. M., S. F.], Osaka City University Medical School, Osaka 545-8585, and Department of Gastrointestinal Surgery, Osaka City General Hospital, Osaka 534-0021, Japan [M. H.]

INTRODUCTION
Carcinogenesis is a multistep process, and carcinoma progresses as the result of accumulated genetic alterations that cause the cell to escape the normal controls on cell growth and differentiation (1–3). Mutation of the $p53$ tumor suppressor gene is a major step in carcinogenesis and the most common genetic defect known to occur in diverse human carcinomas (4–6). One of the biochemical functions of $p53$ protein, which is tightly linked with its tumor-suppressor activity, is its ability to activate transcription, and mutations of the $p53$ gene often abolish this activity (6).

Esophageal carcinoma is a relatively common malignant neoplasm worldwide, and 300,000 new cases of esophageal carcinoma arise each year (7). The frequency of $p53$ gene mutations in human esophageal carcinoma is variable (ranging from 38% to 69%), and no general conclusions have been reached with respect to the relationship between the $p53$ gene status of esophageal carcinoma and its clinicopathological findings (7–12).

$p53$ gene mutations have been detected by screening with anti-$p53$ antibodies for overexpression of the mutant protein in tumor cells (13, 14) and DNA structure-based screening (6, 15). The former is reasonably sensitive and easily detects mutant $p53$ protein. Although a generally good correlation between overexpression and mutation has been reported, numerous exceptions have been described, and the relationship between overexpression and mutation is now thought to be indirect (6, 11). DNA structure-based screening techniques include PCR-SSCP2 (6) and denaturing gradient gel electrophoresis (15). Although these methods are sensitive and can directly detect $p53$ gene mutations, they do not assess the actual function of the $p53$ gene.

To detect $p53$ gene mutation frequency and the functional activity of the encoded gene in human ESCC, we used a $p53$ yeast functional assay described previously (6). This method has several advantages compared with previous methods: (a) it is simple, sensitive and convenient; (b) it disregards silent $p53$ gene mutations that do not affect its function; (c) it detects mutations over most of the $p53$ gene (codons 67 to 346) in one procedure; (d) loss of heterozygosity can be detected easily; (e) the findings of this method are easily recognized as red (mutant-type) or white (wild-type) yeast colonies; (f) the site of mutation can be determined by recovering plasmid DNA from the colonies; and (g) small tumor samples can be analyzed for mutation.

In the present study we screened for $p53$ gene mutations

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1 To whom requests for reprints should be addressed, at Second Department of Surgery, Osaka City University Medical School, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan. Phone: 81-6-6645-3841; Fax: 81-6-6646-6057; E-mail: m5144423@msic.med.osaka-cu.ac.jp.

2 The abbreviations used are: PCR-SSCP, PCR single-strand conformation polymorphism; ESCC, esophageal squamous cell carcinoma.
using a p53 yeast functional assay and analyzed the correlations between p53 gene status, mutation spectrum, and clinicopathological findings in ESCCs.

**MATERIALS AND METHODS**

**Tissue Collection.** Fifty-seven patients with ESCC (44 men and 13 women; ages ranging from 47 to 85 years, mean 64 years) underwent radical esophagectomy with regional lymph node dissection before any anticancer therapy between February 1993 and September 1998 at the Second Department of Surgery, Osaka City University Medical School and the Department of Gastrointestinal Surgery, Osaka City General Hospital. The surgery was curative, without macroscopic regional carcinoma, in all of the patients. Fifty-seven specimens obtained from these patients were examined for p53 gene mutations. A portion of the tissues was fixed in 10% formalin and a portion of the tissues was frozen in liquid nitrogen immediately after surgical resection and stored at −80°C with subsequent RNA extraction. The formalin-fixed, paraffin-embedded tissue blocks were sectioned for histopathological analysis.

**RNA Extraction and Reverse Transcription-PCR.** Tissue samples were obtained from 100 mg of tumor tissue in the specimen. Total RNA was extracted from the sample using an RNA kit, Isogen (Nippon GENE, Toyama, Japan). p53 cDNA was synthesized at 42°C for 50 min using 200 units of Superscript II (Life Technologies, Inc., Tokyo, Japan) from 2 µg of total RNA in 20 µl of reverse transcriptase buffer containing 25 pmol of p53-specific primer, RT-1 (5′-CGGGAGGTAGAC-3′), 10 mM DTT, and 0.5 mM deoxynucleotide triphosphates and heated to 70°C for 15 min and quickly chilled on ice. Then p53 cDNA was amplified using Hot Start PCR (Thermal Cycler Model 2400; Perkin-Elmer, Chiba, Japan) in a 20-µl reaction mixture containing 2 µl of the reverse transcriptase reaction product, 10× reaction buffer, 1.25 units pfu DNA polymerase (Stratagene, La Jolla, CA), 10% DMSO, 50 mM deoxynucleotide triphosphates and heated to 95°C for 1 min and 35 cycles of 95°C for 30 s; 65°C for 60 s; and 72°C for 80 s. After amplification, the PCR products were incubated at 72°C for 2 min. The PCR products were separated on a 1.8% agarose gel to confirm amplification.

**Plasmids.** The yeast expression vector pSS16 (16) was digested with excess amounts of HindIII and SstI, which introduced a gap between codons 67 and 346. The amputation stump was dephosphorylated using calf intestinal alkaline phosphatase (Takara, Otsu, Japan) and the linearized plasmids were separated on a low-melting temperature agarose gel and recovered.

**p53 Yeast Functional Assay.** The crude PCR products and the linearized p53 expression vector pSS16 were cotransfected into the yeast reporter strain yIG397 (6), which contains an integrated plasmid with the ADE2 gene under the control of a p53-responsive promoter. The transfected yeast cells were plated on minimal medium minus leucine and adenine and grown for 48 h at 30°C. When the strain was transformed with a plasmid encoding wild-type p53, the cells expressed ADE2, grew normally, and formed white colonies. Cells containing mutant p53 failed to express ADE2 and formed red colonies, which were smaller than normal because of the accumulation of an intermediate in adenine metabolism. Because the p53 expression vector was linearized at codons 67 and 346, the assay tested the entire DNA-binding domain (6, 16).

In the present study, the p53 yeast functional assay was performed according to the method modified by Tada et al. (17). Yeast was cultured in 150 ml of YPD medium supplemented with 200 µg/ml of adenine, until the OD600 reached 0.8. The cells were pelleted, resuspended in 10 ml of a LiOAc solution containing 0.1 M lithium acetate, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA NaOH, pelleted again, and resuspended in 500 µl of the LiOAc solution. For each transformation, 50 µl of yeast suspension were mixed with 1–5 µl of the unpurified p53 cDNA PCR product, 100 ng of linearized plasmid, 5 µl of sonicated single stranded salmon sperm DNA (11 mg/ml), and 300 µl of LiOAc containing 50% polyethylene glycol 4000 (Wako, Osaka, Japan). The mixture was incubated at 30°C for 30 min and heat-shocked at 42°C for 15 min. The yeast was then pelleted and resuspended with 100 µl of the supernatant and plated on synthetic minimal medium minus leucine plus adenine (5 µg/ml). This was incubated for 48 h in a 30°C humidified chamber. Red colonies were clearly identifiable at this stage, but the color was more intense after 1 additional day at 4°C. More than 200 colonies were counted, and the indices of positive for red colonies were calculated as percentages based on the total number of colonies.

**Assessment of p53 Yeast Functional Assay.** When the rate of red colonies was >20%, the sample was considered positive for a p53 gene mutation (17). When the rate of red colonies was <10%, the sample was considered negative for a p53 gene mutation and was regarded as the background for this assay, as described previously (2, 6). This background represents the PCR-induced errors or/and the presence of an alternatively spliced p53 mRNA (18). Samples, in which the rate of red colonies were from 10 to 20%, were considered positive for a p53 gene mutation only when sequencing analysis confirmed the presence of a clonal mutation.

**Recovery of p53 Plasmids from Yeast and DNA Sequencing.** For each sample that yielded ≥10% red colonies, the plasmid DNA from at least 4 red yeast colonies was extracted and sequenced to make a final decision concerning mutations. The yeast cells were digested with Zymolyase 100T (Seikagaku Corporation, Tokyo, Japan), and the p53 expression plasmids were extracted using a plasmid extraction kit (TOYOBO CO., Osaka, Japan) and transformed into an XL-1 blue Escherichia coli by electroporation and incubated at 37°C overnight on LB ampicillin plates. Plasmid DNA were recovered from cultured E. coli, purified, and sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer) according to the manufacturer’s protocol.

The sequencing primers were as follows: P3seq, (5′-ATTTGATGCTGTCCCCGGACGATATTGAA-3′); P11seq, (5′-TACTCCCCGCTCCCTCAAAAGATG-3′); P12seq, (5′-TTGCGTGAGTATTTGGAGT-3′); P13seq (5′-GCCCATCTCACATCATCACACT-3′).

**Clinical Findings.** For the histopathological staging of the disease, the 1997 TNM classification was used (19). The
histopathological findings of the 57 carcinomas were classified according to the WHO histological typing system (20).

We divided the patients into two groups according to age. The Young Group included those who were <65 years of age, and the Elderly Group consisted of those ≥65 years of age.

The history of smoking and alcohol intake of the patients was obtained from a preoperative personal interview. Alcohol and smoking statuses were also categorized as Baron et al. (21) described. Using alcohol equivalents (150 ml of wine, 330 ml of beer, 180 ml of sake, and 30 ml of hard liquor), drinks/week was divided into three categories as follows: (a) moderate-drinkers, <35 drinks/week; (b) heavy-drinkers, 35–59 drinks/week; and (c) very heavy-drinkers, ≥60 drinks/week. Smoking status was defined to be a function of frequency (cigarettes/day) and duration (years of smoking) and was categorized as follows: (a) nonsmokers; (b) light-smokers (exsmokers who quit ≥10 years ago or smokers of 1–14 cigarettes/day for <30 years); (c) moderate-smokers (15–24 cigarettes/day regardless of duration, 30–39 years’ duration regardless of amount, 1–24 cigarettes/day for ≥ 40 years, or ≥15 cigarettes/day for <30 years); and (d) heavy-smokers (≥25 cigarettes/day for ≥40 years).

The follow-up of these patients ranged from 3 to 88 months, with a mean of 28 months. We evaluated the follow-up of 54 patients excluding 3 who had died within 1 month of surgery.

Statistical Analysis. Statistical significance was evaluated using Fisher’s exact probability test and the $\chi^2$ test. Survival curves were created using the Kaplan-Meier method, and the statistical significance of differences was calculated by the log-rank test. Differences were considered significant at $P < 0.05$. Statistical analysis was performed with Stat View 5.0 statistical software (SAS Institute, Inc.).

RESULTS

Detection of p53 Gene Mutations. A p53 yeast functional assay was performed on 57 samples from 57 patients with ESCC. A summary of the p53 gene mutations obtained using the p53 yeast functional assay and DNA sequencing was shown in Table 1. The incidence of p53 gene mutations was 43 of 57 (75%). Forty-seven different mutations were detected in the 43 samples.

The spectrum of mutations was as follows: 22 (G:C to A:T) transitions, five of which occurred at a CpG dinucleotide; 5 (A:G to T:C) transversions, and 11 (A:G to C:T) transversions. Of the 47 mutations, 22 (51%) were transitions, 16 (37%) were transversions, and 9 (19%) were deletions or insertions.

Of the 47 mutations, 3 were detected in exon 4, 16 in exon 5, 9 in exon 6, 7 in exon 7, 5 in exon 8, 3 in exon 9, and 4 in exon 10. In total, 37 (79%) mutations were detected in exons 5–8, the most highly conserved region.

Of the 43 samples with mutation, 29 samples were detected in carcinomas from those in the Young Group. Of those 29 samples, 7 samples had null mutations (5 nonsense mutations and 2 deletions).

Of the 57 samples, 5 were thought to have two different populations of mutations. Sample 1 contained two populations of mutations at codons 179 and 275, sample 18 at codons 342 and 348, sample 22 at codons 143 and 144, and sample 47 at codons 238 and 273 (Table 1). Moreover, sample 15 contained two populations of mutations, one of which contained a 74-bp deletion at exon 9, and another that contained a 59-bp insertion between exons 8 and 9 (Table 1).

p53 Gene Dysfunction in ESCC and their Clinicopathological Findings. Differentiation of the samples revealed squamous cell carcinoma; 8 well differentiated, 29 moderately differentiated, and 20 poorly differentiated.

We analyzed the correlations between the presence of p53 gene mutation and the clinicopathological findings (Table 2). The incidence of p53 gene mutations observed in the Young Group and in the Elderly Group were 29 of 31 (94%) and 14 of 26 (54%), respectively. The incidence of p53 gene mutations observed in the Young Group was significantly higher than in the Elderly Group ($P = 0.0007$; Fisher’s exact test). No other clinicopathological factors were associated with p53 gene mutation.

Of the 57 patients, 48 (84%), 7 (12%), and 2 (4%) were moderate, heavy, and very heavy drinkers, respectively. Of the 48 moderate, 7 heavy, and 2 very heavy drinkers, 36 (75%), 7 (100%), and 0 had p53 gene mutation, respectively.

Of the 57 patients, 11 (19%), 1 (2%), 36 (63%), and 5 (9%) were nonsmokers, light-, moderate, and heavy smokers, respectively. The smoking history of four patients was not obtained.

However, the presence of p53 gene mutation was not significantly associated with alcohol or smoking status.

Prognosis. Survival rate after surgery was not significantly associated with the presence of p53 gene mutation (Fig. 1; $P = 0.480$, log-rank). We also studied which exon or domain had the most important influence on prognosis, but no association could be found.

In the Young Group, the number of samples without a p53 gene mutation was too small for a statistical analysis of the survival rate after surgery. However, in the Young Group with p53 gene mutation, patients with a null mutation had a significantly shorter survival period than did those without a null mutation (Fig. 2; $P = 0.0455$, log-rank). In the Elderly Group, there was no correlation between the mutational spectrum and the survival period (data not shown).

DISCUSSION

In the present study, we found that p53 gene mutations were detected in 75% of human ESCCs. The incidence of p53 gene mutations was high compared with previous studies using the PCR-SSCP method or immunohistochemical staining (7–12). Our results were consistent with a report of Robert et al. (18), who have described the percentage of p53 gene mutations in ESCC using a p53 yeast functional assay. This high incidence of p53 gene mutations is suggested to reflect the high sensitivity of this method, which is the result of several factors: (a) a large region of p53 cDNA (codons 67–346) can be examined; (b) p53 gene mutations can be detected when the incidence of mutant-type p53 cDNA is >6% in a mixture of wild- and mutant-type p53 cDNA (2), therefore, substantial contamination with normal tissue does
not interfere with this assay; and (c) the simple red/white readout means that mutations are not easily overlooked. In addition, we detected p53 gene mutations in the following regions not covered by routine PCR-SSCP analysis: three in exon 4, three in exon 9, and four in exon 10. This may partly account for the high incidence of p53 gene mutations.

There is a wide variety of p53 gene mutations, the majority of which occur at any one of several hundred potential bp sites in the p53 gene (1). Either exogenous DNA-damaging agents or spontaneous endogenous molecular events cause specific changes with respect to the position and type of mutation in the p53 gene (1, 22). Prior studies have suggested that mutations do not occur randomly. The mutational spectra are being used to explore clues to the etiology gained by classical epidemiological studies (4, 5, 7). Therefore, the mutational spectrum has been the subject of much interest. There are well-known examples of exposure to a particular carcinogen causing specific mutations, and there has been a specific mutation identified in the following types of carcinomas: (a) a codon 249 Arg→Ser mutation in ≅50% of hepatocellular carcinomas from individuals exposed through their diet to hepatocarcinogenic aflatoxins (5, 23); and (b) a codon 249 Arg→Met mutation in ≅25% of lung carcinomas from uranium miners, which is associated puta-

### Table 1

<table>
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<tr>
<th>Sample no.</th>
<th>Tumor diagnosis</th>
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<th>Stage</th>
<th>Functional assay (% red colonies)</th>
<th>Exon</th>
<th>Codon</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
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<td>IIa</td>
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<td>7 249 AGG→AGT</td>
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<td>Arg→Ser</td>
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tively with radon gas exposure (24). In all carcinomas, ~25% of $p53$ gene mutations are transitions occurring at CpG dinucleotides (25). However, in esophageal carcinoma, the frequency of G:C to A:T base transitions at CpG, which represent the predominant $p53$ gene mutations observed in other gastrointestinal carcinomas such as colon carcinomas, is low (4). The mutational frequency (11%) of the CpG site in the present study was in agreement with a previous report (22). The G:C to A:T base transitions at CpG dinucleotides are thought to result from the spontaneous deamination of 5'-methyl cytosine, resulting in C to T replacements as a result of endogenous cellular mistakes (1, 4, 5).

Tobacco and alcohol consumption are the major known risk factors in the pathogenesis of esophageal carcinoma (26). Tobacco and alcohol are thought to make the mucosa susceptible to carcinoma formation, resulting in the acquisition of genetic mutations (27). Moreover, anatomical considerations suggest that the combined effects of tobacco and alcohol may have a greater carcinogenic effect in the esophagus (26). A strong association between tobacco and alcohol consumption and the presence of $p53$ gene mutation has been reported (26). However, in the present study, we found that the presence of $p53$ gene mutation was not significantly associated with tobacco or alcohol consumption. The respective role of each risk factor is difficult to assess because most of the patients with esophageal carcinoma were exposed to both tobacco and alcohol. It was reported that the high prevalence of G:C→T:A transversions in lung carcinoma has been associated with tobacco consumption (1, 4, 22). On the other hand, it has been reported that the mutational spectrum for tobacco and alcohol consumption is diverse and is not predominated by the tobacco-related G→T transversion (22).

In the present study, the G→T transversion was found in 11% of the $p53$ gene mutations. G to A transitions are often found in patients with a smoking history (28). This may be caused by methylating nitrosamines contained in tobacco (28). G to A transitions result from DNA alkylation at deoxyguanosine and the mispairing of O6-methyldeoxyguanosine with thymine (29). A recent study indicated that G to A transitions are the predominant type of point mutation in human esophageal carcinomas (29), but in the present study, only five (11%) were detected. In the present study, we did not find any correlation between the $p53$ mutational spectrum and tobacco and alcohol consumption. Therefore, tobacco and alcohol consumption might be associated with mutations of another oncogene or/and antioncogene rather than with the $p53$ tumor suppressor gene.

Because the mean age of the patients diagnosed as ESCC was 64 years, the patients aged <65 years were classified as the Young Group and the others as the Elderly Group. In the present study, the incidence of $p53$ gene mutations was significantly different between the Young and the Elderly groups, suggesting that the pathways of esophageal carcinogenesis may differ between these two groups. Moreover, the incidence of $p53$ gene mutations decreased with age; the <60 age group, 60–70 age group, and ≥70 age group had 15 of 17 (88%), 18 of 25 (72%), and 10 of 15 (67%) $p53$ gene mutations, respectively. We also examined the prognosis of the Young Group. In the Young Group with $p53$ gene mutation, those who had a null mutation for $p53$ gene had a significantly poorer prognosis than those who did not. One possible explanation for this observation is that null mutations generally cause a loss of the COOH-terminal domain of $p53$ protein, which plays an important role in carcinogenesis (30). This suggests that, in the Young Group, $p53$ gene mutation might cause esophageal carcinogenesis, and that the $p53$ gene might play several important roles that are all lost in those with a null mutation, thus causing an especially poor prognosis. No clinicopathological factor except for age was...
significantly associated with the incidence of p53 gene mutations.

In summary, a p53 yeast functional assay is a highly sensitive, simple, and convenient method for detecting p53 gene mutations compared with previous methods. There may be different esophageal carcinogenesis pathways between the Young and the Elderly groups because the incidence of p53 gene mutations is different between the two groups. In addition,
the null mutation for \( p53 \) gene is a significant prognostic factor in the Young Group with ESCC, but it cannot be detected by immunohistochemistry.

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Detection of \( p53 \) Gene Mutations in Human Esophageal Squamous Cell Carcinomas Using a \( p53 \) Yeast Functional Assay: Possible Difference in Esophageal Carcinogenesis Between the Young and the Elderly Group

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