p53 Alterations but No Human Papillomavirus Infection in Preinvasive and Advanced Squamous Esophageal Cancer in Italy

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Abstract

Geographic differences in exposure to suspected carcinogens have been identified in esophageal carcinogenesis, and both p53 alterations and human papillomavirus (HPV) infection have been reported in esophageal squamous carcinoma (ESC) from high-risk areas, including China and South Africa. The status of p53 alterations and HPV infection in ESC has not been determined in northern Italy, where the incidence of ESC is low. Formalin-fixed paraffin-embedded esophageal samples containing normal, dysplastic, and carcinomatous tissue from 18 patients were examined for p53 protein accumulation with immunohistochemistry, p53 mutation (exons 5–8) with PCR-single-strand conformation polymorphism analysis and DNA sequencing, and HPV accumulation with immunohistochemistry, p53 mutation from 18 patients were examined for p53 protein tissue samples from other geographic areas. Examination of the reports detected in 9 of 18 carcinomas, a finding consistent with mutations were rare in dysplastic lesions but were both precancerous and carcinomatous lesions. p53 alterations and HPV infection in ESC has not been determined in northern Italy, where the incidence of ESC is low. Formalin-fixed paraffin-embedded esophageal samples containing normal, dysplastic, and carcinomatous tissue from 18 patients were examined for p53 protein accumulation with immunohistochemistry, p53 mutation (exons 5–8) with PCR-single-strand conformation polymorphism analysis and DNA sequencing, and HPV infection with PCR using general primers to amplify the LI gene. Accumulation of p53 protein was observed in both precancerous and carcinomatous lesions, p53 mutations were rare in dysplastic lesions but were detected in 9 of 18 carcinomas, a finding consistent with reports from other geographic areas. Examination of the p53 mutation spectrum revealed no hot spot mutation. In contrast, HPV was not found in any of these 18 cases. This is consistent with the findings from other low ESC risk areas in which HPV infection may not play a crucial role in esophageal oncogenesis, whereas the high risk of ESC in China and South Africa may be attributed to frequent HPV infection.

Introduction

In Italy, the annual age-adjusted incidence of esophageal carcinoma was less than 15 cases/100,000 population during 1983–1987 (1). Similar incidence rates were also observed in most other European countries, Japan, and the United States (1). In contrast, a high risk of ESC was reported in China, South Africa, and Iran, with annual age-adjusted incidence rates of more than 50 cases/100,000 population (2). The vast majority of esophageal cancers are of squamous type, located in the upper segment of the esophagus, and characterized by a very poor prognosis. The overall 5-year survival is no higher than 10% (3).

The geographic and ethnic differences worldwide in the incidence of ESC support the hypothesis that esophageal carcinogenesis is a multifactorial process in which attributable factors may differ in different areas (2, 4, 5). Tobacco and alcohol have been associated with increasing esophageal cancer risk in European countries, Japan, and the United States (2, 4–6). In contrast, the greater risk of esophageal cancer in China, Iran, and South Africa has been attributed to dietary factors (2, 5). As in certain other epithelial tumors, multistage oncogenesis involving a progression from normal tissue to dysplasia, to intraepithelial neoplasia, and finally invasive cancer has been described in ESC (7). It has been reported that p53 gene mutations and HPV infection are both detected in precancerous and carcinomatous stages of esophageal carcinogenesis in certain regions, notably China and South Africa (8–12).

Wild-type p53 functions as a transcription factor regulating cell proliferation at the G1–G0 checkpoint (13). Alterations in p53 are said to be involved in the onset and/or progression of ESC (9, 14). The mutation spectrum of the p53 gene has been associated in many cancers with exposure to specific carcinogens (15). Mutations at G:C bp (mainly G:C to A:T transitions) have frequently been detected in ESC in China (Linxian), France (Lyon), and the United States (South Carolina; Refs. 11, 16, and 17). Mutations predominantly at A:T bp have been reported in cases from Brest, France (18).

Inactivation of wild-type p53 function by the E6 protein of HPV has also been suggested to contribute to the pathogenesis of tumors arising from squamous epithelia, including the esophageal mucosa (19, 20). In particular, HPV16, 18, 31, 33, and 51 have been associated with a high risk of neoplastic onset (21). However, the fact that a high frequency of HPV infection has been observed only in China (8) and South Africa (22) casts doubts on HPV’s potential role as an etiological agent of ESC in the low-risk western countries.

In the present study, normal, dysplastic, and neoplastic esophageal samples were examined at histological, molecular
genetic, and/or immunohistochemical levels for HPV infection and p53 alterations. The aims of this study, in a series of patients from a low-risk area for ESC, were to determine (a) any role of HPV infection in ESC in Italian patients; (b) the association between p53 alterations and ESC; and (c) the timing of HPV infection and p53 alterations in the multistage process of esophageal carcinogenesis.

**Materials and Methods**

**Patients.** Formalin-fixed paraffin-embedded tissue blocks from 18 patients (all males: 50–72 years old; mean age, 61 years) who underwent esophagectomy for invasive ESC (stage I, 1 patient; stage II, 10 patients; stage III, 5 patients, and stage IV, 2 patients) were selected from the archives of the Department of Pathology, at the University of Padova, in northeastern Italy. Tumor staging was according to the TNM system (23). No patients received irradiation or chemotherapy prior to surgery, which can induce p53 protein overexpression (24). Normal (nondysplastic), dysplastic, and carcinomaous samples were available from the same or different tissue blocks. The distance separating any foci of ED from invasive ESC ranged from 0.4 to 4.5 cm. ED and ESC were histologically assessed on the basis of standardized criteria (25). ED was classified as low or high grade. A three-level grading system (G1, G2, and G3) was applied to ESC. A scoring system [four for koilocytosis (vacuolation), 2 for binucleation and multinucleation, and 1 for dyskeratosis, intraepithelial capillary loops, basal cell hyperplasia, or acanthosis] according to Toki and Yajima (26) was used to score the cytohistological lesions. A combined score higher than 5 was considered as indicative of HPV infection (26).

**p53 IHC.** Dewaxed 5-μm formalin-fixed paraffin-embedded sections were immersed in citrate buffer (0.01 M sodium citrate, pH 6) and boiled in a microwave oven at 600 W for 10 min. After blocking endogenous peroxidase activity with 0.3% H2O2 in methanol for 30 min, sections were incubated at 25°C overnight with a 1:100 dilution of the monoclonal antibody Pab 1801 (NovoCastra, Newcastle, United Kingdom) against wild-type and mutant forms of p53 protein. The avidin-biotin complex procedure was used according to the manufacturer’s directions (Vector Laboratories, Burlingame, CA). For negative control purposes, the same procedure was followed except that the primary monoclonal antibody was replaced with PBS. A colon cancer sample with known p53 mutation and protein accumulation was used as a positive control. In each specimen, p53 protein accumulation was scored as positive (immunoreactivity in >5% of examined nuclei) or negative (<5%).

**DNA Extraction.** Seven serial sections (1 × 5-μm, 5 × 10-μm, and 1 × 5-μm thick) were cut from formalin-fixed paraffin-embedded esophageal samples containing normal, dysplastic, and neoplastic tissue. The first and last 5-μm sections were routinely stained with H&E to confirm the representative areas and were used as a guide for microdissection. The selected areas were microdissected from the unstained slides and were combined in a 1.5-ml microcentrifuge tube for deparaffinization and proteinase K digestion (27). DNA was extracted by adding one-third volume of saturated NaCl followed by ethanol precipitation, as described previously (28). DNA concentration was quantified on the TKO-100 Minifluorometer according to the manufacturer’s instructions (Hoefer Scientific Instruments, San Francisco, CA). A negative control consisting of all reagents but no tissue section was carried out for each DNA extraction.

**PCR.** The primers for amplification of p53 exons 5–8 and conditions for PCR have been reported elsewhere (28). Briefly, a total of 25 μl of PCR mixture [75 mM Tris base (pH 9.0), 20 mM ammonium sulfate, 0.01% Tween 20, 1.5 mM MgCl2, 200 μM deoxyribonucleoside triphosphates, 0.5 μM each of primers, and 0.5 units of thermostable DNA polymerase (Advanced Biotechnologies, Surrey, United Kingdom)] containing 20 ng of DNA template was prepared for a 40-cycle amplification. The reaction was carried out in the Perkin-Elmer 9600 automated thermal cycler (Perkin Elmer-Cetus). A negative control with no DNA template was run in parallel for each amplification.

General primers (GP5 and GP6) reported by Snijders et al. (29) were used to amplify the L1 genes of HPVs 6, 11, 16, 18, 31, 32, and 33 and under low PCR stringency utilizing primer annealing at 40°C. An HPV-infected cervical sample was used as a positive control. Gastric tissue was selected as an HPV-negative control. A housekeeping gene, α2-macroglobulin exon 24 (176 bp; primers: 5'-TCTATGTACTGGATTATCTA-3’ and 5'-CTAAGCTAAGTATCATATAA-3’), was coamplified with L1 genes of the HPVs (139–154 bp) to confirm that failure to detect HPVs was not a result of severe DNA degradation, which is frequently encountered in archival materials. Amplification for both L1 genes and exon 24 of the α2-macroglobulin gene was carried out in a 25-μl PCR mixture as described for the p53 gene. The reaction was carried out as follows: denaturation at 94°C for 5 min in the first cycle followed by a 30-s annealing at 40°C, 2-min extension at 72°C, and 30 s at 94°C for a total of 40 cycles of PCR. The extension of the final cycle was increased to 5 min to ensure complete extension. The PCR products were resolved by 2% agarose gel electrophoresis and were evaluated after ethidium bromide or silver staining (Bio-Rad, Richmond, CA).

**SSCP.** SSCP mobility shifts of PCR fragments of p53 exons 5–8 resulting from mutations were determined under the SSCP conditions described elsewhere (28). In summary, a mixture of 1 μl of PCR product and 5 μl of denaturing buffer (95% formamide, 20 mM disodium EDTA, 0.05% xylene cyanol, and 0.05% bromphenol blue) were heated to 95°C for 5 to 10 min and then immediately placed on ice to prevent renaturation. Samples (5 μl) of each denatured PCR product were loaded onto 0.75-mm 12% polyacrylamide gels with 22.5 mM Tris-borate (pH 8.4) and 2 mM EDTA in the Miniplate II Slab Cell (Bio-Rad). Electrophoresis was carried out at ambient temperature at a constant 100 V (4–6 h).

Single-stranded DNAs were demonstrated with silver stain (Bio-Rad) according to the manufacturer’s instructions. A known mutant control and a wild-type PCR fragment were resolved along with samples in the same polyacrylamide gel for direct comparison. Samples with mobility shifts were verified by a second independent PCR-SSCP analysis.

**DNA Sequencing.** Shifted bands of p53 exons 5–8 detected by SSCP were punched out with a pipette tip and reamplified for an additional 35 cycles using 1 unit of Promega Taq polymerase (Promega, Madison, WI). Enriched PCR fragments were cloned into pCRII vector using the TA cloning kit (Invitrogen, San Diego, CA). PCR-SSCP was performed for selected clones side-by-side with the original PCR fragment to verify mobility shifts. Plasmids with the correct insert were subjected to DNA sequencing using the Taq Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). DNA fragments were tagged with fluorescent-labeled deoxyribonucleotides and resolved in a Model 370A Automatic DNA Sequencer (Applied Biosystems). The CircumVent Thermal Cycle Sequencing kit...
Table I  p53 alterations in multistage esophageal tumorigenesis

<table>
<thead>
<tr>
<th>Case</th>
<th>Normal</th>
<th>Dysplasia</th>
<th>Carcinoma</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IHC</td>
<td>SSCP</td>
<td>IHC</td>
</tr>
<tr>
<td>1</td>
<td>–</td>
<td>+ (LG)</td>
<td>+ (G1)</td>
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<tr>
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<td>+ (G3)</td>
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<td>17</td>
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<td>– (HG)</td>
<td>+ (G1)</td>
</tr>
<tr>
<td>18</td>
<td>–</td>
<td>– (HG)</td>
<td>– (G1)</td>
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</tbody>
</table>

a = negative; +, positive.

*LG, low grade; NT, not tested; HG, high grade; G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated.

Results

p53 Alterations. Accumulation of p53 protein was observed in 2 of 18 (11%) nondysplastic, 9 of 18 (50%) dysplastic, and 12 of 18 (67%) carcinomatous samples (Table I). p53 immunoreactivity was detected only in nuclei (Fig. 1). p53 mutations were detected in 1 of 16 (6%) dysplastic and 9 of 18 (50%) carcinomatous samples. Representative p53 mutations in DNA sequencing analysis are illustrated in Fig. 2. No mutation was found in nondysplastic lesions (Table I). Among neoplastic lesions, p53 protein accumulation with no gene mutations was found in six cases.

Mutation in both dysplastic and neoplastic lesions was observed in case 15. Point mutations, including two cases of G:C to T:A transversion, two cases of A:T to G:C transition,

Fig. 1. High-grade squamous esophageal dysplasia: p53 immunoreactive nuclei (monoclonal antibody Pab I 801) are detectable through the whole thickness of squamous epithelium (case 10). X75.

(New England BioLabs, Beverly, MA) was used as an alternative DNA sequencing procedure. Sequencing of PCR products using α-35S-labeled dATP and sequencing gel electrophoresis were carried out as described elsewhere (28).

NISH. Five-μm-thick formalin-fixed paraffin-embedded sections of nondysplastic, dysplastic, and carcinomatous samples were mounted on pretreated slides (Enzo Diagnostic, Farmingdale, NY) and dried at 40°C for 2 days. HPV s 6/11, 16/18, and 31/33/51 were examined with the Patho-Gene kit (Enzo Diagnostic) in which biotin-labeled DNA probes are designed to hybridize with the E6-E7 open reading frame of HPVs. An HPV-infected uterine cervical sample was used as a positive control. A negative control was obtained by omitting the biotin-labeled DNA probes in hybridization.

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p53 and HPV in Esophageal Carcinogenesis

One case of C:G to T:A transition, and two cases of A:T to T:A transversion, were the major types of gene alteration. Examination of the coding regions revealed no site-specific hot spot mutations. Comparison of the p53 mutation spectrum in ESC between countries where tobacco and alcohol are associated with ESC risk (France, Italy, Japan, and the United States) and where ESC risk is associated with dietary factors (China) revealed no significant difference (Table 2).

**HPV Infection.** No cases reached a final score higher than 5 in the morphological evaluation of HPV infection. NISH for HPVs (6/11, 16/18, and 31/33/51) also failed to show any positive reaction in nondysplastic, dysplastic, and carcinoma-tous samples, whereas positive nuclei were always observed in a positive control. When PCR (a more sensitive technique) was performed, both the L1 gene of HPV from the positive control and the α1-macroglobulin housekeeping gene were readily amplified, but no trace of HPV DNA could be detected in the esophageal samples.

Table 3 shows the varying prevalence of HPV infection in ESC documented in the literature. In addition to our observations, the absence of HPV infection in ESC has also been reported in Chiba (Japan) and in Sweden. When the frequency of HPV infection in ESC was compared with the prevalent ESC risk factors identified in each geographic area, the presence of HPV in ESC was significantly higher in countries where ESC is associated with dietary and nutritional factors (China, Iran, and South Africa) than in areas where it is associated with tobacco and alcohol (France, Italy, Japan, Sweden, and the United States; \( P < 0.0001, \chi^2 \) test).

**Discussion**

We detected p53 mutations in more than 50% of ESC from a population in northeastern Italy where tobacco and alcohol are documented risk factors for this disease. The high frequency of p53 mutation is consistent with reports from several other geographic areas (11, 14, 17, 18, 30–42). Mutation of the p53 gene is rare in dysplastic lesions but the fact that this mutation was detected in both dysplasia and carcinoma in one case (case 15) suggests a monoclonal expansion of cells with p53 mutations during esophageal oncogenesis or supports the hypothesis that clones with p53 mutations may acquire a proliferative advantage over non-mutated cells. Although p53 mutation has rarely been detected at a molecular genetic level in precancerous lesions, p53 accumulation was sometimes detected using immunohistochemistry in both nondysplastic mucosa and dysplastic lesions. p53 immunodetection in the early stages of ESC oncogenesis has already been reported in the literature, mainly in the basal layer of noninfiltrative lesions (9, 12, 43). The nature of such an immunohistochemical finding is not yet clear, and it has been suggested that mutations take place primarily in the basal epithelial layer, spreading later.

**Table 2** p53 mutations in ESC by prevalent risk factors identified in different geographic areas

<table>
<thead>
<tr>
<th>Attributable risk</th>
<th>Area</th>
<th>Deletion N</th>
<th>Insertion N</th>
<th>GC to AT Cpg</th>
<th>GC to AT Non-Cpg</th>
<th>GC to TA</th>
<th>GC to TA</th>
<th>GC to CG</th>
<th>GC to CG</th>
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<td>2</td>
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<td>0</td>
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<tr>
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<td>0</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>3</td>
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<td>7</td>
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<td>6</td>
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</table>

\[ P^{*} = 0.26 \text{ (Fisher's exact test among point mutations; specific mutation versus others).} \]
p53 gene between geographic areas with different (prevalent) N3-adenine or N7-guanine, DNA adduct formation, or 8-OH-
throughout the mucosa as the grade of dysplasia increases
ISH, a 

The absence of any differences in mutation spectrums in the 
02- or 04-thymine (47, 48), were also detected in two cases. 
A:T to T:A transversion, which can result from alkylation of 
provide clues to exposure to specific carcinogens (15). In this 
eration (44). 

It has been suggested for host response to neoplastic cell prolif-
eration (44). 

It has been proposed that p53 mutation spectrum may 
provide clues to exposure to specific carcinogens (15). In this 
study, we observed two cases of G:C to T:A and two cases of 
A:T to T:A transversion, which can result from alkylation of 
N3-adenine or N7-guanine, DNA adduct formation, or 8-OH-
guanine oxidative damage (45, 46). A:T to G:C transitions, 
which can be induced by deamination of adenine or alkylation 
of O2- or O4-thymine (47, 48), were also detected in two cases. 
The absence of any differences in mutation spectrums in the 
p53 gene between geographic areas with different (prevalent) risk factors suggests that a similar mechanism resulting in p53 
mutation may operate among populations with different types of 
environmental exposure. In addition, p53 mutations in ESC 
showed no codon-specific hot spot in this or other studies (15), 
suggesting that multiple carcinogens are involved in ESC carcino-
genesis.

The role of HPV in ESC has been questioned (35). In the 
current study, we detected no HPV infection in any stages of ESC 
oncogenesis by morphological evaluation, NISH, or PCR. This 
finding is consistent with observations from several low-risk 
countries, where HPV was never or rarely detected in ESC (35, 38, 39) and contrasts with findings from high-risk countries (e.g., China and South Africa) where a high frequency of HPV infection has been detected in ESC (8, 40, 41). A highly significant association of HPV infection with geographic areas (China, Iran, and South Africa) suggests that dietary and nutritional factors may be re-
ponsible for frequent HPV infection in ESC.

The different sensitivity of the techniques and differences in 
the likelihood of HPV being detected (according to which

segment of the HPV open reading frame is chosen for screening) 
have been cited as possible contributors to the difference in 
the frequency of HPV infection detected in different geographic 
areas (49, 50). HPV can exist in two forms (i.e., integrated and 
episomal) in host cells (51). During integration, a conserved 
region, comprising the E6-E7 genes, randomly inserts into the 
host genome (52). In this study, HPV screening was performed 
using an E6-E7 probe in NISH and an attempt was made to 
 amplify the L1 gene by PCR: since both techniques failed to 
detect HPV infection, any lack of analytical sensitivity and 
specificity of the methods adopted can be ruled out.

p53 mutations are frequently detected in ESC worldwide, 
including Italy. p53 protein accumulation precedes p53 mutation 
in multistage ESC oncogenesis. In Italy (a low ESC risk area), the role of HPV infection is negligible in ESC carcino-
genesis, whereas HPV infection may contribute to the high 
incidence of ESC in China and South Africa.

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