Human Papillomavirus DNA and Protein in Tissue Samples of Oesophageal Cancer, Barrett’s Oesophagus and Oesophagitis

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Abstract. Background: The risk of presenting with oesophageal cancer is associated with Barrett’s oesophagus, with a higher prevalence in some Asian and African countries. Human papillomavirus (HPV) DNA has been identified in oesophageal carcinomas, which share common features with cervical cancers and originate in stratified epithelium. Materials and Methods: Sixty-eight paraffin-embedded tissue biopsies were selected from Mexican patients: 17 from oesophageal cancers, 28 from cases of Barrett’s oesophagus and 23 from cases of oesophagitis. HPV protein was detected immunohistochemically and the presence and types of HPV DNA were assessed by polymerase chain reaction. Results: HPV DNA-positive results were found in 26% of samples of oesophagitis, 96% of samples of Barrett’s oesophagus and 88% of samples of oesophageal cancers. HPV viral types 6 and 11 were prevalent. HPV protein was detected in 41 samples (60%). Conclusion: Mexico has a high prevalence of HPV in premalignant and malignant oesophageal diseases compared with other countries.

The human papillomavirus (HPV) is a small virus that affects stratified epithelia. At least 80 different types have been identified and classified according to their effects on cutaneous and mucosal tissues. Most cause proliferative benign lesions or warts, but some sub-types can cause premalignant and malignant lesions (1). The most frequent malignant lesions occur in the female genital tract, causing cervical carcinoma, the most common cancer in the world (2).

Oesophageal cancer is one of the main causes of death associated with disorders of the digestive tract. Its aetiology has a marked geographical variation, with the highest incidences in China, South Africa and Iran (3). The most frequent oesophageal neoplasias are squamous cell carcinomas and adenocarcinomas (4,5).

The risk of developing oesophageal cancer is associated with the previous presence of dysplasia on Barrett’s oesophagus. This is a premalignant lesion observed histologically in the distal oesophagus and related to gastro-oesophageal reflux disease (GERD) (6).

Morphological and immunohistochemical studies first identified the presence of HPV in papillomas (7) and oesophageal squamous carcinomas (8,9). The incidence of HPV detection in these tumours varies widely from zero to 71% (10). These divergences are probably caused by variations in the techniques employed and in the geographical populations studied (high or low risk) (3,11). The evidence thus strongly suggests a causal role for HPV in oesophageal cancers. The HPV forms found more frequently in oesophageal lesions are types 16 and 18, using polymerase chain reaction (PCR) as the gold standard for detection of viral DNA.

The purpose of this study was to test for the presence of HPV protein and DNA sequences in paraffin-embedded Barrett’s oesophagus, carcinoma and oesophagitis tissues to corroborate the presence of HPV and to identify the viral types present in these oesophageal diseases.

Materials and Methods

We studied 68 oesophageal tissue biopsies, which were stored fixed in paraffin blocks. They were selected retrospectively as follows: 17 oesophageal cancers, 28 cases of Barrett’s oesophagus and 23 cases
of oesophagitis. This selection was performed using the registries of the Pathology Department from the Specialities Hospital of the National Western Medical Center in Mexico.

Each of the tissue biopsies was studied immunohistochemically, using an indirect immunological test for viral proteins, and by PCR to demonstrate the presence of HPV DNA.

**Polymerase chain reaction.** Extraction of DNA in all the samples followed the Wright-Manos method (12-14). Tissue was excised from the paraffin block using a scalpel. The fragments were placed in a 2-mL Eppendorf tube and mixed with 1 mL N-octane (ACROS Organics, Fisher Scientific Division, Pittsburgh PA, USA) at room temperature for 30 minutes to dissolve the paraffin. It was then centrifuged for 3-5 minutes at 14,000 rpm, the supernatant was removed using a pipette, then 500 µL of 100% ethanol was added, mixed and centrifuged at 10,000 rpm for 5 minutes. The supernatant ethanol was removed, two or three drops of acetone were added and the tubes were left open at 37 °C until the solvents evaporated.

After the deparaffinization process, 100 µL digestion buffer (50 mM Trizma base, pH 8.5, 1 mM EDTA, 0.5 % Tween 20) containing 200 µg/mL of proteinase K solution (Fungical Invitrogen, Carslbad, CA, USA) was added and the tube was incubated at 55 °C for 3 hours or overnight at 37 °C. The tubes were then microcentrifuged for 3 seconds to remove excess liquid, incubated at 95 °C for 10 minutes to inactivate the proteinase K, re-centrifuged for 30 seconds and stored at -20 °C. After centrifugation at 10,000 rpm, the tubes were opened and stored at 4 °C. Products were separated in TBE 1 x buffer using 12% polyacrylamide gel electrophoresis. Initial electrophoresis conditions were 120 v for 10 minutes followed by 150 v for 3.5 hours for total DNA separation. Molecular weight markers were a 10 bp DNA ladder and pBR 322 digested with Hae III. To determine the molecular weights of the resulting fragments and to compare the band patterns obtained according to the restriction maps for each viral type, we took at least two fragments from those paired in the centres and edges of the gel (15). Contamination during the different procedures was avoided using appropriate controls and procedures (16).

**Immunohistochemistry.** Anti-peroxidase (PAP) immunohistochemistry on deparaffinized and rehydrated sections was used to detect HPV proteins. Endogenous peroxidase activity is quenched by incubating the specimen for five minutes with Peroxidase Block. The specimen is then incubated for 15 minutes with the prepared biotinylated primary antibody, followed by a 15-minute incubation with streptavidin-peroxidase. Tissues were incubated sequentially with primary anti-HPV antibodies (polyclonal rabbit, anti-bovine Papillomavirus [BPV-1] code No. B9580, DAKO Corporation, Carpinteria, CA, USA).

Antibody complexes were visualized following a 5-minute incubation with 3,3’-diaminobenzidine (DAB) + substrate-chromogen which results in a brown-colored precipitate at the antigen site, according to the manual of the DAKO Animal Research Kit.

The PCR and immunohistochemistry results were classified into three groups based on patient sex, age, diagnosis and clinical stage at the time of the diagnosis. We also considered pathology, diet and toxicology.

**Statistical analysis.** Data were analysed using SPSS 10.0 for Windows. The results are presented as means and 95% confidence intervals (CI 95%) based on standard deviations for quantitative variables and as percentages for qualitative data, using Pearson and logistic regression coefficients with odds ratios (OR). Epi-info version 6.04 software was used for ANOVA. Classification of the percentage of viral types and diagnosis of immunohistochemistry were performed manually.

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### Table I. DNA amplification by PCR.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial concentration</th>
<th>Final concentration</th>
<th>Volume/20 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer PCR</td>
<td>10 x</td>
<td>1 x</td>
<td>2 L</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>50 µL</td>
<td>1.5 µL</td>
<td>0.6 µL</td>
</tr>
<tr>
<td>dATP*</td>
<td>10 µL</td>
<td>0.2 µL</td>
<td>0.4 µL</td>
</tr>
<tr>
<td>dCTP*</td>
<td>10 µL</td>
<td>0.2 µL</td>
<td>0.4 µL</td>
</tr>
<tr>
<td>dATP*</td>
<td>10 µL</td>
<td>0.2 µL</td>
<td>0.4 µL</td>
</tr>
<tr>
<td>dTTP**</td>
<td>10 µL</td>
<td>0.2 µL</td>
<td>0.4 µL</td>
</tr>
<tr>
<td>CPI**</td>
<td>10 µL</td>
<td>1 µL</td>
<td>2 µL</td>
</tr>
<tr>
<td>CPHG**</td>
<td>10 µL</td>
<td>1 µL</td>
<td>2 µL</td>
</tr>
<tr>
<td>Taq</td>
<td>5 U/µL</td>
<td>2.5 U/100 µL</td>
<td>0.1 µL</td>
</tr>
</tbody>
</table>

*Deoxyribonucleoside triphosphates.
**Oligonucleotide pairs. These primer combinations can identify more than 90% of HPV types 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 56 and 58.

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c) Sample of each amplification = cocktail 8.3 µL + 9.7 µL H₂O + 2 µL DNA = 20 µL.
A drop of mineral oil was added to each tube to prevent evaporation. After centrifugation at 10,000 rpm, the tubes were subjected to 32 cycles of PCR using an initial step of 94 °C for 5 minutes; denaturation at 94 °C for 30 seconds; 51 °C for 30 seconds; at 72 °C for 1 minute, followed by a final extension at 72 °C for 1 minute. PCR products were stored at -4 °C. Products of the 188 pairs of fractions were separated by 6% polyacrylamide gel electrophoresis and stained with silver nitrate (15).

**Viral type identification.** DNA fragments were recovered from the gel using a sterile scalpel placed in 0.5-mL Eppendorf tubes and incubated for 20 minutes at 95 °C with 50 µL recuperaion solution (10 mM Tris-HCl pH 9.0, 5 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100). Aliquots of 5 µL were used to re-amplify the DNA and 10 µL aliquots of the re-amplified product were used to corroborate the second amplification. Subsequently, 10 µL aliquots of the re-amplified product were mixed with the restriction enzyme Rsa1, using the manufacturer’s recommended protocol (Gibco BRL; Life Technologies, Gaithersburg, MD, USA). Products were separated in TBE 1x buffer using 12% (19:1) polyacrylamide gel electrophoresis. Initial electrophoresis conditions were 120 v for 10 minutes followed by 150 v for 3.5 hours for total DNA separation. Molecular weight markers were a 10 bp DNA ladder and pBR 322 digested with Hae III. To determine the molecular weights of the resulting fragments and to compare the band patterns obtained according to the restriction maps for each viral type, we took at least two fragments from those paired in the centres and edges of the gel (15). Contamination during the different procedures was avoided using appropriate controls and procedures (16).
In Table II we present age, sex and cases where the HPV DNA was present, according to the different oesophageal diseases (See Figures 1 and 2).

The major HPV viral types detected by PCR in the 36 positive samples were strains 6 and 11, while less frequent were strains 18, 31, 33, 51 and 58 (See Figure 3).

Correlation analysis showed statistically significant associations between the presence of HPV DNA and male gender and consumption of tobacco and alcohol. Logistic regression analyses showed significant associations between tobacco consumption alone and the presence of oesophageal cancer (OR 6.2, CI 1.2-33.1, p<0.05), and the presence of the viral genome in individuals with Barrett’s oesophagus (OR 24.39, CI 3.0-196.9, p<0.01).

**Results**

**Table II.** Age, sex and HPV DNA presence in the different oesophageal diseases.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Non-specific oesophagitis (n=23)</th>
<th>Barrett’s oesophagus (n=28)</th>
<th>Oesophageal cancer (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>54.6 ± 16.09 (CI 47.65 to 61.57)</td>
<td>58 years ± 16.05 (CI 51.7 to 64.12)</td>
<td>63.06 ± 15.61 (CI 55.03 to 71.09)</td>
</tr>
<tr>
<td>Sex</td>
<td>Males 12 (52%) Females 11 (48%)</td>
<td>Males 18 (64%) Females 10 (36%)</td>
<td>Males 15 (88%) Females 2 (12%)</td>
</tr>
<tr>
<td>HPV DNA presence</td>
<td>6 (26%)</td>
<td>27 (96%)</td>
<td>15 (88%)</td>
</tr>
</tbody>
</table>

*HPV DNA and immunohistochemistry.* Immunohistochemistry was positive in 41 samples (60%) and negative in 27 (40%). The presence of HPV DNA correlated strongly with the type of pathology: oesophagitis, Barrett’s oesophagus and cancer (rP 0.56; p=0.000). All the oesophageal samples were tested and a golden-brown staining in the cell cytoplasm was considered as positive (See Figure 4).

There was only a weak association between the presence of HPV DNA and positive immunohistochemistry for the viral protein (rP 0.20; p 0.09).
Discussion

Adenocarcinoma is related to Barrett’s oesophagus, mainly in cases of severe dysplasia, but little is known about the role viruses play in the aetiology of oesophageal cancers. HPV has been identified in some of these patients (17-19). We also identified HPV proteins in the stratified squamous epithelia adjacent to adenocarcinomas.

The main HPV types described in carcinomas of the oropharynx are 6, 11, 16, 18, 31, 33 and 73 (2). We also found these forms, but HPV type 16 was less frequent. The presence of a single virus was rare and we observed various types in cases of cancer and Barrett’s oesophagus: 6, 11, 18, 31, 35 and 58. Types 6 and 11 were the most prevalent in this series.

Age had no significant effect, even though it was expected to be a significant factor. In the correlation analysis, the presence of HPV alone was a risk factor and smoking and alcohol consumption were associated, as is well documented (2) Logistic regression analysis showed that the risk of developing cancer was not affected by the presence of HPV DNA, but was affected by smoking and drinking habits. In patients with Barrett’s oesophagus, we found that the only statistically significant risk factor was the presence of HPV DNA. In patients with oesophageal cancer, the presence of HPV DNA as well as tobacco consumption was also significant ($p=0.005$).

According to the medical literature, the prevalence of HPV varies from 10% to 60%, depending on the methods used and population type. The average prevalence rate detected by
PCR in patients with oesophageal cancer is 15.2% (17). In this study, we detected HPV DNA in 88% of oesophageal cancer samples and in 96% cases of Barrett's oesophagus, which is higher than reported in the literature. We cannot conclude that the presence of HPV leads to oesophageal dysplasia, but it is clearly an important risk factor.

References