A series of 30 surgical resection materials of patients with squamous cell carcinoma (SCC) and 10 with adenocarcinoma (AC) were analyzed histologically and by polymerase chain reaction (PCR) to demonstrate whether human papillomavirus (HPV) has a role in the etiology of esophageal cancers. Epithelial changes suggesting HPV infection were histopathologically detected in 19 of 30 (63.3%) SCC and in 4 of 10 (40%) AC cases. By PCR, HPV type 16 and/or type 18 DNA were detected in 10 of 30 (33.3%) SCC and HPV type 18 DNA alone was detected in 1 of 10 (10%) AC cases. Histopathologic findings related to HPV infection were detected in all the cases with positive HPV DNA. A significant correlation was present between histopathologic positivity and PCR positivity. The positivity rate of HPV infection was higher in well-differentiated SCC cases when compared to poorly-differentiated ones. These results suggest that HPV might have a role in the etiology of esophageal cancers and histopathologic findings of HPV infection were reliable markers for the presence of HPV DNA in tissue.

**Key Words:** Esophagus cancer, Human Papillomavirus

**Introduction**

The cause of esophageal cancer is unknown. However, epidemiologic studies in several areas of the world suggest a relationship with alcohol, tobacco, nitrosamines, vitamin deficiencies, aflatoxin, candidal and viral infections (1,2).

The incidence of esophageal cancer shows certain geographic variations. South African countries, Iran, China, India, Ceylon and Puerto Rico are high incidence areas. In the United States, the incidence is 4-5 fold higher in blacks than in whites. Male:female ratio is 3:1 in low incidence areas. This ratio is characteristically lower in high incidence areas (1,3-6).

Infections caused by certain viruses have a role in the pathogenesis of some cancers in humans. However, it has been proposed that viral infection alone does not result in cancer (7). It has been argued that cancer could occur either when viral proteins were expressed in an inappropriate cell type or in an immunocompromised host (7).

In recent years, the existence of histopathological changes related to HPV infection, HPV antigens by immunocytochemically and HPV DNA by DNA detection techniques was shown in esophageal cancer tissue and adjacent mucosa from the patients in high incidence areas (4-6,8,9). It has been proposed that persistent HPV infection in the esophageal epithelium could destroy the immunologic resistance mechanisms and initiate a multistep mechanism ending in esophagus cancer (10).

In this study, we examined epithelial changes in both cancer tissue and adjacent mucosa associated with HPV infection in SCCs and ACs. We also examined the existence of HPV DNA types 16 and 18, which are known to be a high risk group for carcinoma development in epithelial tissues by polymerase chain reaction (PCR) technique.

**Materials and Methods**

Formalin fixed and paraffin embedded tissue samples derived from 30 SCC and 10 AC cases were included in this study. As a control group, 10 healthy subjects (5 male and 5 female) participated in this study after signing a consent form. Control samples were taken from the esophagi of 10 control subjects with endoscopically normal upper gastrointestinal systems. Differentiation degrees of cancer tissues were determined and epithelial changes related to HPV infection were examined in the border of lesion-normal tissue (6,8) by Hematoxylin-Eosin staining. Scoring was performed as shown in Table 1.
HPV infection is diagnosed when 6 points or more are allocated (6).

After histopathologic evaluation, the presence of HPV Type 16 and 18 DNA was examined by using modified PCR (11). Five 5 (µm-thick sections were obtained from each paraffin block and placed in a 500 µl Eppendorf tube. The sections were deparaffinized by adding 200 µl of xylene and centrifugation. Then xylene was decanted, its residue was removed by adding 99.5% ethanol and this procedure was repeated. Afterwards, TRIS hydrochloride buffer solution (10 mmol/L pH 7.5) was added to each sample. The specimens were digested at 37°C overnight with proteinase K (Sigma Chemical Co, St Louis, MO) at 100 (g/ml extraction buffer (TRIS hydrochloride, 10⁻² mmol/L pH 7.5; ethylenediamintetraacetic acid 1 mmol/L pH 7.9 and 0.5% sodium dodecylsulfate). Later proteinase K was inactivated by heating at 100 °C for 10 minutes.

After DNA extraction, PCR was performed. In brief, 2 µg of purified DNA from each sample was added to the PCR mixture (ammonium sulfate, 16.6 mmol/L; TRIS HCl, 67 mmol/L, pH 8.8 at 25°C; magnesium chloride, 6.7 mmol/L; β mercaptoethanol, 10 mmol/L; EDTA 6.7 µmol/L and bovine serum albumin, 170 µg/ml). Type specific primers for HPV 16 and 18 of 0.1 µmol/L, deoxyribonucleotide triphosphate (deoxyadenosine triphosphate, deoxythymidine triphosphate of 1.5 mmol/L each) and 2.5 U Taq DNA polymerase were added to the tubes. Mineral oil of 40 µl was added to each sample to prevent evaporation. All samples were then put in the Thermal Cycler and pre-incubated 2 minutes at 94°C for denaturation. Thirty-five cycles of amplification were performed according to following procedure.

1 minute at 94°C for denaturation,
2 minutes at 55°C for annealing,
1.5 minutes at 72°C for amplification.

After amplification, size marker was put in the first well, positive control sample in the second well, negative control sample in the third well and 10 µl of amplified DNA from each tissue sample in the other wells. Then all materials were electrophoresed on a 3% agarose gel in a concentration of TRIS 90 mmol/L, EDTA of 2 mmol/L at pH 8 and boric acid of 90 mmol/L, and were made visible by ultraviolet illumination after staining with 1% ethidium bromide.

The positive control samples were kindly provided by Dr. E.M. de Villers (DKFZ, Heidelberg-Germany). The sequences of the primers that were prepared for E6-E7 region of the HPV genome and amplified product sizes are listed in Table 2.

Statistical analysis between two groups was performed by using Fischers exact (FE) test, and among three groups by Kruskall-Wallis variance analysis. For in-group analysis, Wilcoxon signed rank (WSR) and for correlation analysis, Spearman-rho (SR) tests were used.

Results
Thirty SCC patients were included in our study. Seventeen (56.6%) of the patients were female and 13 (43.4%) were male. Six (60%) of 10 AC patients were male and 4 (40%) were female. In the healthy control group, 5 (50%) subjects were male and 5 (50%) were female.

Table 1. HPV score system for histological diagnosis of HPV infection.

<table>
<thead>
<tr>
<th>Histological Parameters</th>
<th>Score</th>
</tr>
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<tbody>
<tr>
<td>Koilocytosis</td>
<td>4</td>
</tr>
<tr>
<td>Bi and multi-nucleation</td>
<td>2</td>
</tr>
<tr>
<td>Dyskeratosis</td>
<td>1</td>
</tr>
<tr>
<td>Intra-epithelial capillary loops</td>
<td>1</td>
</tr>
<tr>
<td>Basal-cell hyperplasia</td>
<td>1</td>
</tr>
<tr>
<td>Acanthosis</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HPV</th>
<th>Nucleotide Sequences</th>
<th>Product Size (Base Pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-16</td>
<td>5'-CCCAGCTGTAATCATCGATGGAGA-3' 3'-ACCTTCTGGACAATTACCCGTGTG-5'</td>
<td>253</td>
</tr>
<tr>
<td>HPV-18</td>
<td>5'-CGACAGGAAAGACTCCAACGA-3' 3'-TCAATTTACTGTTGAAATGCTG-5'</td>
<td>201</td>
</tr>
</tbody>
</table>
By histopathologic evaluation, HPV score was 6 or above (positive) in 19 of 30 (63.3%) SCC and 4 of 10 (40%) AC cases (Figure 1). There was no significant difference between males and females in the rate of histopathological HPV infection (WSR: p>0.05).

HPV type 16 and/or 18 DNA was detected in 10 of 30 SCC (33.3%) and 1 of 10 AC tissue samples (10%) using the PCR technique (Figure 2). There was no significant difference between the two groups (FE: p>0.05).

Nineteen of 30 SCC tissue samples (63.3%) were histopathologically well-differentiated, 5 (16.6%) were moderately differentiated and 6 (20%) were poorly differentiated. HPV infection was histopathologically positive in 14 of 19 well-differentiated SCCs (73.6%), 4 of 5 moderately differentiated SCCs (80%) and 1 of 6 poorly differentiated SCCs (16.6%). Although there was no significant difference between well-differentiated and moderately differentiated SCC cases, histopathological
HPV infection rate was significantly higher in well-differentiated SCCs than poorly differentiated ones (WSR: p<0.05).

HPV 16 and/or 18 DNA was detected as positive in 6 of 19 (31.5%) well-differentiated SCCs (HPV type 16 DNA in 3, HPV type 18 DNA in 1 and both of them in 2 samples), 3 of 5 (60%) moderately differentiated SCCs (all were both HPV type 16 and 18) and 1 of 6 (16.6%) poorly differentiated SCC cases (both HPV type 16 and 18). Statistical comparisons between these groups showed no significant difference (KW: p>0.05).

Five (50%) of 10 AC tissue samples were well-differentiated, 1 (10%) was moderately differentiated and 4 (40%) were poorly differentiated. Histopathological HPV infection was detected in 2 of 5 (40%) well-differentiated AC, 1 of 1 (100%) moderately differentiated AC and 1 of 4 (25%) poorly differentiated AC tissue samples. HPV type 18 DNA was detected in only 1 (10%) well-differentiated AC case. In the adenocarcinoma group, statistical analysis could not be done because of the small number of cases.

HPV infection was not found in control group samples either by histopathological examination or by PCR technique. Results are summarized in Table 3.

Histopathologic findings related to HPV infection were detected in all the patients in whom HPV DNA was positive. A significant correlation was present between the histopathologic positivity and PCR positivity (SR: r = 2.23, p< 0.001).

### Discussion

Esophagus cancer is found to be the ninth most frequent neoplasm of all the neoplasms. This malignant tumor has a certain geographical distribution (1,3). It is more frequently seen in males. In high incidence areas, the male to female ratio is low. The great geographical variations in the incidence of esophagus cancer have been explained by viral etiology 3,10,12,13.

HPVs are oncogenic viruses and show oncogenic activity through spoiling mucosal immune resistance and destroying tumor suppressor genes (7,10). HPV 16 is the most common type and responsible for 50% of genital HPV infections (15). It has been shown that oncogenic HPV types, especially HPV type 16 and 18, could immortalize normal human cells through two early gene products (E6 and E7 proteins) and play an important role in the cancers of several body sites (14).

There is increasing evidence suggesting that HPV infection (primarily with HPV type 16 and 18) is an important etiological factor in human genital tract cancers (15, 16-28). HPV was also found to be associated with both benign and malignant lesions developed in many other sites of the body including the skin, aero-digestive tract, oral cavity, and colorectal and anal region (14,29-42).

The geographical variations of a certain tumor can imply a possible viral etiology. It has been reported that the prevalence of HPV DNA in certain head and neck tumors shows notable geographical differences. For example, studies of oral cancers from India and Taiwan showed a high incidence of HPV (74% and 76%
respectively), whereas those from Western Europe and the United States showed lower incidence (ranging from 10 to 17%). In countries such as China and India, the chewing of betel quid is proposed as a primary cause of carcinogenesis for oral cancers. The high incidence of HPV DNA in these cancers suggested a synergistic interaction between HPV and chemical carcinogens (34).

There are also several reports suggesting HPV infection is a possible risk factor in the development of esophageal carcinoma (5,8,10, 43-45).

Chang et al. demonstrated the presence of HPV DNA (mainly HPV 16 and 18) in esophageal dysplasia and invasive carcinoma in 4 distinct studies from a high-risk area of China (12,46,48,50). Bjorge et al. found an increased risk for subsequently developing esophageal cancer among HPV 16 sero-positive healthy blood donors (49).

Chen et al. also demonstrated HPV positivity in 60% of the SCC cases from a high-risk area of China (4). Williamson et al. also reported HPV DNA positivity in 71% of esophagus cancer tissue and/or adjacent normal mucosa (13).

The results of our study are in good agreement with the above-mentioned ones. In our study, we found histological HPV infection in 63.3% of SCC and 40% of AC tissue samples. Additionally, we detected HPV 16 and/or 18 DNA in 33.3% of SCCs and 10% of ACs. These results suggest a role of HPV in the development of esophagus cancer.

While HPV DNA detection rate was found to be high in precancerous lesions and well-differentiated cancers, it was found to be low in poorly differentiated ones (6,37,41). Similarly, we found HPV infection in well-differentiated SCCs significantly higher than in poorly differentiated ones (73.6% versus 16.6%). The high detection rates of HPV infection in precancerous lesions may imply a possible role of HPV in the transformation of a benign lesion (e.g. metaplasia) to dysplasia and neoplasia.

HPV infection of the esophagus was first suggested in 1982 by Syrjanen, who described the cytopathic changes of HPV in esophageal cancer specimens (44). Subsequently, the existence of HPV in benign and malign esophageal lesions was demonstrated by other researchers. Therefore, HPV has been presented as an etiological agent in the pathogenesis of esophageal cancer. However, HPV DNA positivity has varied considerably in different studies (4,9,12,46-52).

The frequency of esophageal HPV infection shows geographical variations like esophageal carcinoma. For example, Munoz et al. found that HPV infection and premalignant lesions of the esophagus were higher in Linxian, a high-risk area, for esophagus cancer, than those in Jiaoxian, a low-risk area in China (53).

In the esophagus, the development of an invasive carcinoma is likely to be a multi-step process and persistent exposure to both viral and chemical agents can synergistically cause malignant lesions 10,12. The animal model of esophage cancer developing from the result of interaction between papillomaviruses (Bovine Papillomavirus Type 4) and immuno-suppressive agents (bracken fern) was proved in cattle (6).

The East Anatolia region, where this study was performed, is on the same geographical line as other high-risk areas for esophagus cancer, such as China and Iran. Unfortunately, there is no reliable, population-based study reflecting the incidence of esophagus cancer in this area. However, in two distinct studies carried out in our gastroenterology department, the incidence of esophagus cancer was reported to be higher in the East Anatolia region than in other parts of Turkey. Additionally, the male:female ratio was found to be 1.6:1 in these studies (54,55). As mentioned above, the male:female ratio tends to be lower in high incidence areas and has been quoted as 1.3-1.5:1 (13). These results support the opinion that the East Anatolia region is a high-risk area for esophagus cancer.

The reasons for the high incidence of esophagus cancer in the East Anatolia region can partly be explained by social-economic status, prolonged exposure to aflatoxin (because of food storage during long winters) and hot beverage drinking habits. Our study implied the role of HPV infection in the possible high incidence of this cancer.

In some studies, HPV DNA could not be found in esophageal carcinoma tissue samples. Paz, who investigated 167 head and neck cancers and demonstrated HPV in 25 of them (15%), was not able to detect HPV DNA in 11 esophageal cancer tissue samples (34). Similarly, Kiyabu found HPV DNA positivity in anogenital cancers and oropharyngeal cancers in varying proportions, but he was not able to detect HPV DNA in 13 esophageal cancers (56). Loke from Hong Kong, Benamouzig from France, Lagergren from Sweden,
Talamini from Italy, and Poljak from Slovenia also did not confirm an association between HPV and esophageal cancers (57-61). Although their results seem to be contradictory to our results, their study group was smaller and the geographic area they studied was not a high-risk area for esophageal cancer.

Although HPV types tend to infect squamous epithelium, it has also been shown in benign and malignant lesions of several mucosal sites that do not contain squamous epithelium such as the colon, lung, and paranasal sinuses (18,38,39,46).

We found histopathological HPV infection in 4 of 10 (40%), and HPV 18 DNA in 1 of 10 (10%) ACs. However, statistical analysis could not be done because of the small number of cases. These results may suggest that HPV had a role not only in SCCs but also in ACs.

We found histopathological HPV infection in all HPV DNA positive cases, but not vice versa. This finding is not surprising because we investigated only 2 of approximately 90 HPV DNA types. Perhaps these rates could be found to be higher by using consensus primers. However, most HPV types were not found to be associated with malignant lesions. Therefore, we examined high risk HPV DNA types 16 and 18 to demonstrate the role of these viruses in carcinogenesis. Our results, along with those of other researchers, showed the high sensitivity of these changes in HPV infection (4,6). However, further studies are needed to demonstrate the speciality of histopathological changes for HPV infection.

I may be assumed that esophagus cancer may develop as a result of a complex interaction between known carcinogens and biological promoters such as HPV. The immunological resistance of the host determines the end-point of this interaction (53,55).

Finally, we believe that HPV infection, especially with high risk HPVs (HPV type 16 and 18), has an etiological role in both esophageal SCC and AC through several mechanisms.

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