

## Detection of Epstein-Barr virus DNA by polymerase chain reaction in surgical specimens of patients with squamous cell carcinoma of the larynx and vocal cord nodules

Skumöz hücreli larenks kanseri ve vokal kord nodüllü hastaların cerrahi doku örneklerinde polimeraz zincir reaksiyonuyla Epstein-Barr virüsü DNA'sı araştırması

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**Objectives:** We investigated the incidence of Epstein-Barr virus (EBV) infection in surgical specimens of squamous cell carcinoma of the larynx and vocal cord nodules.

**Patients and Methods:** Formalin-fixed, paraffin-embedded tissue samples randomly selected from 22 patients with squamous cell carcinoma of the larynx (20 males, 2 females; mean age 52 years) and from 17 patients with vocal cord nodules (14 males, 3 females; mean age 38 years) were examined by the polymerase chain reaction. The patients were assessed in terms of localization of the disease, smoking habits, duration of smoking, and the presence or absence of EBV DNA.

**Results :** Twenty-one patients with laryngeal carcinoma and nine patients with vocal cord nodules were smokers. Polymerase chain reaction showed EBV DNA in 11 patients (50%) with laryngeal carcinoma and in seven patients (41.2%) with vocal cord nodules. No significant differences were found with respect to EBV DNA positivity, smoking habits, smoking period, the number of cigarettes consumed daily, localization of disease, and tumor stage ( $p > 0.05$ ).

**Conclusion:** Epstein-Barr virus does not seem to be directly associated with the pathogenesis of laryngeal squamous cell carcinoma, but its role in the etiology of laryngeal proliferative diseases needs more elucidation.

**Key Words:** Carcinoma, squamous cell/virology; DNA, neoplasm; DNA, viral; Herpesvirus 4, human/isolation & purification; laryngeal neoplasms/immunology; polymerase chain reaction/methods; vocal cords/immunology.

**Amaç:** Larenjeal skuamöz hücreli karsinom ve vokal kord nodüllerine ait cerrahi örneklerde Epstein-Barr virüsü araştırıldı.

**Hastalar ve Yöntemler:** Rastgele seçim yöntemiyle skuamöz hücreli larenks kanseri olan 22 hastanın (20 erkek, 2 kadın; ort. yaş 52) ve vokal kord nodülü olan 17 hastanın (14 erkek, 3 kadın; ort. yaş 38) formalin ve parafinde bekletilen cerrahi doku örnekleri polimeraz zincir reaksiyonu yöntemiyle incelendi. Olgular, hastalığın yerleşim yeri, sigara içme alışkanlığı, süresi ve miktarı ve EBV DNA varlığı açısından değerlendirildi.

**Bulgular:** Larenks kanserli olguların 21'inde, vokal kord nodüllü olguların dokuzunda sigara içme öyküsü vardı. Polimeraz zincir reaksiyonu ile, kanserli olguların 11'inde (%50), vokal kord nodüllü olguların yedisinde (%41.2) EBV-DNA pozitifliği saptandı. Gruplar içinde EBV-DNA varlığı, sigara içme alışkanlığı, süresi, bir günde tüketilen sigara sayısı, hastalığın yerleşimi ve tümör evresi bakımından anlamlı farklılık görülmedi ( $p > 0.05$ ).

**Sonuç:** Bulgularımız, larenjeal skuamöz hücreli karsinom patogenezi ile EBV arasında doğrudan ilişki olmadığını göstermektedir; ama larenjeal proliferatif hastalıkların etyolojisindeki rolü daha fazla araştırma gerektirmektedir.

**Anahtar Sözcükler:** Karsinom, skuamöz hücreli/viroloji; DNA, neoplazi; DNA, viral; Herpesvirüs 4, insan/izolasyon ve purifikasyon; larenks neoplazileri/immünoloji; polimeraz zincir reaksiyonu/yöntem; vokal kord/immünoloji.

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Laryngeal carcinoma accounts for 25% to 40% of all head and neck malignancies. The role of many factors, especially tobacco use and alcohol consumption has been the subject of research for laryngeal carcinoma.<sup>[1]</sup> It is known that certain viruses have oncogenic potential. The relationship between laryngeal carcinoma and viruses has recently received considerable interest.<sup>[2]</sup>

Epstein-Barr virus (EBV), which is a member of Herpesviridae family, is a double-stranded DNA virus. Nearly all individuals in underdeveloped countries get infected by EBV during childhood. However, infection-related symptoms generally remain unnoticed as they are mild. It is estimated that EBV affects 80% to 90% of adult population in the world.<sup>[2]</sup>

Epstein-Barr virus is transmitted through saliva and pharyngeal epithelial cells, and B-lymphocytes are a natural site of infection in humans. The oropharynx is the site of primary infection and eventual propagation of EBV, including replication, cell lysis, and sequential cycles of virion oscillation. It has been shown that the parotid and oropharyngeal epithelial cells harbor EBV, providing convenient circumstances for replication of the virus.<sup>[3]</sup> Infection of B-lymphocytes enables the virus to spread to local tissues, distant lymphoid tissues, and to other epithelial cells. Epstein-Barr virus is the only herpes virus that has the ability to change and transform B-lymphocytes into a state of continuous proliferation.<sup>[4]</sup> Transformation of B-lymphocytes results in an immune response with the development of anti-EBV B-lymphocytes.<sup>[5]</sup> So far, there has been growing evidence indicating a close relationship between EBV infection and many diseases including non-endemic Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's lymphoma, peripheral T-cell lymphoma, gastric epithelioma and esophageal ulcer, oral leukoplakia, and anti-EBV antibody associated with the acquired immunodeficiency syndrome.<sup>[2,6,7]</sup>

In this study, we investigated the incidence of EBV infection in surgical specimens of squamous cell carcinoma of the larynx and vocal cord nodules.

## PATIENTS AND METHODS

The study included formalin-fixed, paraffin-embedded tissue blocks randomly selected from patients who underwent surgery for squamous cell carcinoma of the larynx (22 patients; 20 males, 2

females; mean age 52 years) and vocal cord nodules (17 patients; 14 males, 3 females; mean age 38 years).

The patients were assessed in terms of localization and stage of tumors, smoking habits, duration of smoking, and EBV positivity in tumor tissues. The classification and staging of laryngeal tumors were made according to the AJCC 1992 (American Joint Committee on Cancer) criteria.

All samples of squamous cell carcinoma of the larynx and vocal cord nodules were analyzed in two groups depending on the presence or absence of EBV DNA.

A nominal scale was used to evaluate smoking habit (yes-1, no-2), tumor localization (supraglottic - 1, glottic - 2), and nodule localization (right vocal cord - 1, left vocal cord - 2). Comparisons were made with the chi-square test. Mann-Whitney U-test was used for comparisons of phase, smoking period, and the number of cigarettes consumed daily. A *p* value of less than 0.05 was considered significant.

Three or four sections of 5 µm thickness were cut from each paraffin block, deparaffinized with the use of xylene, and then rehydrated with alcohol.

Extraction of DNA was made as described in the literature.<sup>[8]</sup> Briefly, the tissues were first treated with 500 µl of digestion buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 25 mM disodium EDTA (ethylenediaminetetraacetate), and 0.5% sodium dodecyl sulfate] in 10 µl proteinase K stock solution (200 µg/ml final concentration) and were then incubated overnight at 37 °C. Phenol/chloroform extraction was carried out twice. Precipitation of the DNA yield from the aqueous phase was obtained by adding 0.25 volume of 8 M ammonium acetate and an equal volume of isopropanol. Glycogen (10 mg/ml) was also used to increase the DNA yield. After incubation at -20 °C for an hour, the DNA was recovered by centrifugation at 14,000 rpm for 12 minutes. The DNA pellet was rinsed with 70% ethanol and resuspended in 10 µl sterile water. The DNA template was stored at -20 °C until assayed.

Polymerase chain reaction (PCR) was employed to detect EBV DNA with the following primer sets: gp1 (5' GGC TGG TGT CAC CTC TGT TA 3') and gp2 (5' CCT TAG GAG GAA CAA GTC CC 3'). The reaction mixture contained 1.5 µg of the template DNA, 1.25 mM each of four deoxyribonucleoside triphosphates (dATP, dCTP, dTTP, and dGTP), 50

pmole of each primer, Taq buffer, and 1.5 U of Taq polymerase (Promega Corporation, Madison, Wisconsin, USA). The amplification was performed for a total of 45 cycles, which included denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and elongation at 72 °C for 1 min. Positive controls consisted of EBV-positive Raji cells. Negative controls included the reaction mixture with sterile water added in place of the the template DNA. The PCR products were then analyzed on 2% agarose gel (Seakem GTG, FMC Bioproducts, Rockland, ME, USA) stained with ethidium bromide for ultraviolet visualization (Fig. 1).

## RESULTS

In the laryngeal carcinoma group, all the patients but one were smokers. Smoking period ranged from 36 to 540 months (mean 351.4±120.24 months), with 20 to 40 cigarettes daily (mean 23.8±8.04 cigarettes).

Squamous cell carcinoma of the larynx arose from supraglottis in 10 cases (45.5%) and glottis in

12 cases (54.6%), with tumor stages II, III, and IV in two, 12, and six patients, respectively.

Polymerase chain reaction analysis showed EBV DNA in 11 patients (50%) with laryngeal carcinoma, whose tumors were supraglottic in five patients (50%), and glottic in six patients (50%). The tumor stage was I in one patient (50%), III in six patients (50%), and IV in four patients (66.7%). All these patients were smokers, with a smoking period ranging from 360 to 540 months (mean 405.8±73.93 months), and a daily consumption of 20 to 40 cigarettes (mean 23.6±8.09 cigarettes).

Of seventeen patients with vocal cord nodules, nine patients were smokers. Smoking period ranged from 60 to 420 months (mean 190.6±106.05 months), with 10 to 20 cigarettes daily (mean 16.6±5.00 cigarettes).

Polymerase chain reaction analysis showed EBV DNA in seven patients (41.2%) with vocal cord nodules, involving the right vocal cord in four patients (57.1%) and the left vocal cord in three patients (42.9%). Of these seven patients, three (42.9%) were smokers, with a smoking period ranging from 156 to 420 months (mean 252.3±145.98 months), and a daily consumption of 10 to 20 cigarettes (mean 16.6±5.77 cigarettes).

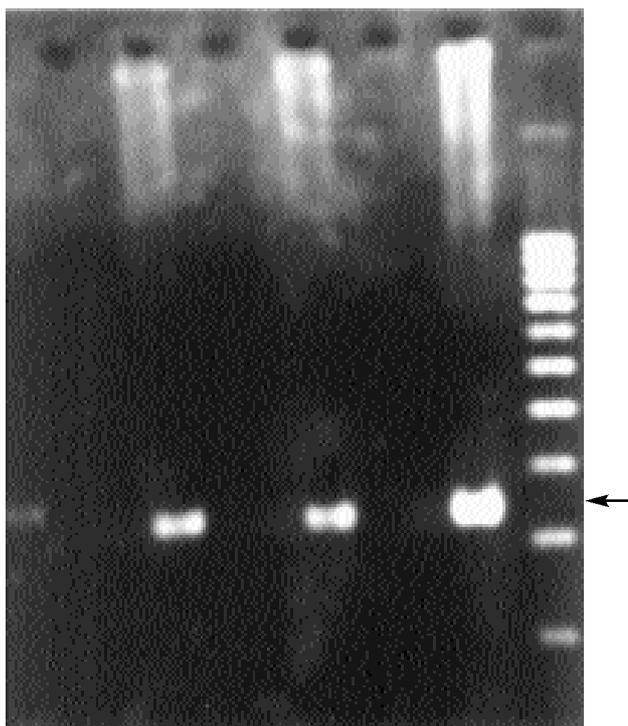
Statistical analyses showed no significant differences with respect to EBV DNA positivity, smoking habits, smoking period, the number of cigarettes consumed daily, localization of disease, and tumor stage ( $p>0.05$ ).

## DISCUSSION

Cancer has a genetic basis at cell level, with many cancers emerging from a single cell. The relationship between cancer and viruses is that viral genes directly affect tumor growth through their effect on cellular genes responsible for growth control.<sup>[9]</sup>

Epstein-Barr virus is a member of human herpes virus family and causes infectious mononucleosis.<sup>[10]</sup> Data from several studies suggest that EBV is involved in the development or progression of squamous cell carcinoma of the nasopharynx, oral cavity, larynx, and esophagus, as well as in gastric epithelioma and Hodgkin's lymphoma.<sup>[11-13]</sup> In addition, it has also been implicated in the etiologies of the African type Burkitt's lymphoma, thymic carcinoma and Sjögren's syndrome.<sup>[14,15]</sup>

The relationship between laryngeal cancers and EBV has been of interest to many authors. Kiaris et



**Fig. 1 -** Polymerase chain reaction (PCR) products in agarose gel electrophoresis. Lane 1- PCR control, a band of 259 bp amplified from HBV DNA. Lane 2,4- PCR negative control with no template DNA. Lane 3,5,6- PCR with primer 1 and primer 2. Lane 6- PCR positive control, a band of 239 bp amplified from the EBV-positive cell line. M-100 bp ladder.

al.<sup>[11]</sup> used PCR analysis and detected EBV in nine (33%) of 27 cases with squamous cell carcinoma of the larynx; however, they found no association with disease stage, histological differentiation, and nodes at pathology. Brichacek et al.<sup>[7,16]</sup> found EBV DNA in three of five patients with supraglottic laryngeal carcinoma, and in six of seven cancers of palatine tonsil with the help of in situ hybridization method. Shu et al.<sup>[6]</sup> detected EBV in 80 of 102 cases with head-neck cancers (53 nasopharynx, 49 other sites) with the use of PCR and Southern blot hybridization. On the other hand, Khabie et al.<sup>[17]</sup> reported that PCR analysis showed no relationship between tonsil squamous cell carcinoma and EBV DNA expression. In our study, although PCR analysis revealed EBV DNA in half of the cases with laryngeal squamous cell carcinoma, no significant differences were found with regard to EBV positivity, localization, stage, smoking period and the number of cigarettes consumed daily.

It should be noted that the PCR technique does not allow to determine the exact settlement of the virus in the tissues and that the possibility of lymphocytes in the tissue cannot be excluded as the source of EBV positivity. In addition, no information is obtained regarding the layer of squamous epithelia on which EBV DNA is recovered.<sup>[18]</sup>

Vocal cord nodules are benign mucosal lesions of the larynx most frequently seen in young women and young boys. They develop as a result of hyperfunction in voice formation, the major factors of which include shouting, talking in noisy environment, and chronic coughs. People who are engaged in using their voice in louder intensity and for long durations on professional grounds (singers, teachers, phone operators, etc.) are more likely to have vocal cord nodules. However, not all people performing such activities suffer from vocal cord nodules, suggesting other factors including those of physiological, medical, and psychological.<sup>[19,20]</sup>

In this study, surgical specimens of patients with vocal cord nodules were initially accepted as controls. Interestingly, EBV DNA was found positive in seven patients (41.2%) in this group, though the difference did not reach significance in terms of the presence of EBV DNA, nodule location, smoking habit, smoking period, and the number of cigarettes consumed.

In conclusion, detection of EBV DNA in 50% and 41.2% of patients with squamous cell carcinoma of the larynx and vocal cord nodules, respectively, may suggest a causal relationship between EBV and the development of laryngeal proliferative diseases.

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