Esophageal Squamous Cell Carcinomas Arising in Patients from a High-Risk Area of North China Lack An Association with Epstein-Barr Virus

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Abstract
The aim of this study was to determine whether EBV associates with esophageal squamous cell carcinoma (ESCC), the most common malignancy in some parts of northern China, because these tumors frequently have an intense lymphocyte infiltrate. Fifty-one paraffin-embedded samples of ESCC from a high-risk area of North China were studied. The tumors included 9 well-differentiated, 31 moderately differentiated, and 11 poorly differentiated tumors. The cancer tissues and their nonmalignant adjacent mucosa (16 dysplastic and 42 normal) were evaluated by in situ hybridization using an antisense EBV-encoded RNA-1 probe and PCR amplification for EBV BamHI W fragment. In all cases, EBV was negative by both in situ hybridization and PCR. Our study suggests that EBV does not play a role in the carcinogenesis of ESCC in the geographic region.

Introduction
EBV infection is important in the development of many human malignancies including Burkitt’s lymphoma, Hodgkin’s disease, and B-cell non-Hodgkin’s lymphoma (1–4). EBV has also been associated with nasopharyngeal and gastric cancers (5–8), and lymphoepithelioma-like carcinomas arising in many sites including stomach, lung, and uterine cervix (9–11). The lymphoepithelioma-like tumors are characterized by the presence of an intense lymphocytic infiltration intimately associated with the tumors. In recent years, some authors have proposed a potential relationship between EBV and ESCC, but their results remain controversial. EBV infections were found in ESCC developing in European patients (12), but not in Japanese patients (13). ESCC is the most common malignancy developing in some parts of northern China, and the cause remains unclear. These squamous cell tumors from northern China tend to be moderately to poorly differentiated lesions, and they are frequently associated with a variably intense lymphocytic infiltrate. Because tumors with lymphocytic infiltrates have an association with EBV infection (14, 15), we postulated that ESCC arising in China would show a significant association with EBV. The current investigation was undertaken to determine whether EBV associates with ESCC in patients living in a high-risk area.

Materials and Methods
Patients and Histopathology Data. Fifty-one esophagectomy specimens with ESCC were studied. All of the patients were diagnosed and treated in Anyang Tumor Hospital in Anyang City, a high-risk area of esophageal cancer in the Henan Province in North China. All of the tumor tissues were fixed by formalin after surgery and were embedded in paraffin. Five-μm sections were cut from the paraffin blocks. One section from each block was stained with H&E and evaluated by three observers. Tumors were classified according to the criteria of the WHO (16). Nonneoplastic adjacent mucosa was available for evaluation in 42 of the 51 tumor specimens.

ISH. A 30mer oligonucleotide was synthesized in the DNA Core Facility of University of Cincinnati and was digoxigenin-labeled using an oligonucleotide tailing kit (Boehringer Mannheim). The oligonucleotide probe is complementary to the small nuclear EBER-1 in latently infected cells. ISH was performed as follows. Briefly, 5-μm-thick sections were deparaffinized, dehydrated, and digested by pronase K at a concentration of 150 μg/ml at 37°C for 15 min. The digested sections were washed in glycine (2 mg/ml), dehydrated through graded ethanol, and air-dried. After 1-h prehybridization, the tissues were hybridized at a concentration of 1.5 ng/μl digoxigenin-labeled probe at 37°C overnight, and the hybridization signal was detected with an anti-digoxigenin-antibody-alkaline phosphatase conjugate for 1 h at room temperature followed by counterstaining with Nuclear Fast Red. We used a known EBV-positive posttransplant lymphoproliferative disorder of the liver as the positive control. The negative control consisted of the sense probe.

DNA Extraction for PCR EBV Detection. Five-μm sections were serially cut from paraffin blocks and stained with H&E. These stained sections were microdissected under a microscope at ×40. Five to ten stained sections were used for each sample depending on the size of the tumor focus. Microdissected tissues were placed in a 1.5-ml microfuge tube and treated with digestion buffer [50 mM Tris- HCl (pH 8.5), 1 mM EDTA, and 0.5% Tween 20] containing 200 μg/ml protease K at 37°C for 24–48 h. Samples were then heated to 95°C for 8–10 min to inactivate the protease K and were centrifuged at 12,000 rpm for 30 min.

PCR. The PCR reactions were performed with primers (EBV1: 5’-CCTTTAGACTGGAAGA-3’, EBV2: 5’-TA-AAGGATACACGACGCAG-3’) specific for the EBV BamHI fragment of the BamHI W region.
The amplification was performed with 50 ng of genomic DNA in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 0.1 μM of each primer, 0.2 mM each dATP, dGTP, dCTP, dTTP, 4% DMSO, and 0.6 units of AmpliTag DNA polymerase (Perkin-Elmer, Norwalk, CT) in a total volume of 25 μl. After an initial hot start at 95°C for 4 min, amplification for EBV was carried out for 30 cycles (1 min at 94°C, 30 s at 55°C, and 1.5 min at 72°C) in a Thermal Cycler (MJ Research, Watertown, MA). The β-globin gene, which was used as a control, was amplified for 35 cycles (1 min at 94°C, 2 min at 52°C, and 3 min at 72°C). Each 5 μl of PCR products was electrophoresed on 1.5% agarose gel and was visualized by ethidium bromide staining. The EBV-positive DNA derived from a patient with a posttransplant lymphoproliferative disorder of the liver was used as a positive control. Samples lacking DNA were used as negative controls for each case.

**Results**

The 51 patients ranged in age from 37 to 74 years (mean age, 55); 33 of the patients were male, and 18 were female. Thirty-four tumors arose in the middle esophagus and 17 arose in the distal esophagus. Among the 51 tumors, 9 were well differentiated, 31 were moderately differentiated, and 11 were poorly differentiated. One tumor was an early carcinoma; the rest were advanced lesions, in which 28 (55%) had lymph node metastases. A severe lymphoid infiltration surrounding tumor tissue was seen in 12 cases (Fig. 1). The adjacent mucosa was dysplastic in 16 patients and normal in 42 patients.

**ISH.** In the ISH survey, most of lymphocytes showed an intense hybridization signal localized to the nuclei of cells in the positive-control tissue (Fig. 2). In all of the 51 ESCC samples, the cancer cells were completely negative for a hybridization signal (Fig. 3). Areas of dysplasia and normal mucosa were also negative, EBER-1 signal was present in only a few of the nonneoplastic lymphocytes surrounding the invasive cancer in three cases.

**PCR.** None of the studied samples amplified with the EBV primers, whereas the β-globin gene was successfully amplified in 50 of 51 cases. Both the EBV and the β-globin gene amplified in the positive control (Fig. 4).

**Discussion**

Squamous cell carcinoma of the esophagus is one of the most common malignancies in northern China. The annual age-adjusted mortality rate is up to 169 per 100,000, and the cumulative death rates are 32% for males and 20% for females in the county of Linxian, Henan province, China (17). The cause of the cancer is still unclear, although various factors have been postulated to be involved in the development of the disease, including intake of foods contaminated with nitrosamine and fungal toxins; nutritional deficiency of vitamins A, C and B, and zinc; chronic use of alcohol and tobacco; the presence of chronic esophagitis; a genetic predisposition (17–19); and high levels of carcinogenic polycyclic aromatic hydrocarbons (20).

Infectious agents have also been implicated in ESCC, particularly the DNA tumor virus HPV. High incidences of HPV positivity have been found in the ESCC specimens from high-risk areas of China, South Africa, Korea, and Alaska (21–23). But, HPV is rarely identified in ESCC in Japan, the United States, the United Kingdom, the Netherlands, and low-risk areas of China (24, 25). Therefore, HPV may play a significant role in esophageal carcinogenesis in those areas of the world with a high incidence of the disease.

Another tumor-related virus, EBV, associates with many human malignancies, including Burkitt's lymphoma, Hodgkin's disease, B-cell non-Hodgkin's lymphoma (1–4), and lymphop epithelioma-like carcinomas (9–11). Evidence of EBV is also found in nasopharyngeal carcinoma and gastric
adeno-carcinoma (5–8). Both nasopharyngeal and gastric cancers are very common in China, and EBV has been identified in Chinese patients with these tumors (26, 27). However, it is unclear whether EBV also plays a role in the carcinogenesis of ESCC. There are only a few reports on this subject in the literature.

One study was unable to find evidence of EBV DNA by a PCR survey of 41 Japanese surgically resected specimens and 12 ESCC cell lines (13). Mori et al. (28) demonstrated EBV infection in one esophageal lymphoepithelioma-like carcinoma by PCR, ISH, and immunohistochemistry but found no evidence of EBV in another 29 ESCC cell lines examined. In another study, 2 of 10 ESCCs in Sweden were found to be EBV-positive by PCR, but all of the cases were negative by ISH and immunohistochemistry (29). The authors concluded that the EBV identified by PCR was derived from contaminating peripheral blood cells. In contrast, EBV infection was found by Jenkins et al. in 8.3% (5 of 60) of ESCC specimens and 6% (1 of 16) of ESCC cell lines, using the DNA extracted from microdissected cells by PCR and Southern blot analyses. The five EBV-positive samples were derived from France (3 of 14) and Italy (2 of 20). The ESCCs from United States and from Iran were EBV-negative (12). These results suggest that EBV may be involved in the development of ESCC in some areas in Europe.

In the present study, the EBER signal was observed in only a small number of lymphocytes infiltrating or surrounding cancer tissue but was negative in all of the normal or dysplastic epithelia and cancer cells by ISH despite the presence of a coexisting intense lymphoplasmacytic response. No EBV sequences were amplified by PCR in any of the 50 cases containing amplifiable DNA of our study. The DNA in this study was extracted from carefully microdissected cells so that EBV contamination in lymphocytes was avoided. Our results indicate that EBV does not play a role in the carcinogenesis of ESCC in the high-risk area of North China.

References


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