Absence of Human Papillomavirus-16 and -18 DNA and Epstein–Barr Virus DNA in Esophageal Squamous Cell Carcinoma

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To elucidate the association of human papillomavirus (HPV) and Epstein–Barr virus (EBV) with carcinogenesis of the esophagus, 41 surgically resected specimens and 12 cell lines of esophageal squamous cell carcinoma were examined for the presence of HPV DNA and EBV DNA by polymerase chain reaction using primers for the E6 regions of HPV-16 and -18 and for the EBNA 1 region of EBV. We designed the reaction condition to amplify HPV and EBV DNA specifically and detected by gel electrophoresis. In ethidium bromide staining, no band was detected either for the HPV E6 region or for the EBV EBNA 1 gene in any of the surgically resected specimens and the cell lines, although the HPV sequence was detectable by Southern blot hybridization, which is a more sensitive detection method than staining; three out of 41 surgically resected specimens were positive for HPV-18 by Southern blot hybridization of polymerase chain reaction products. However, the number of viral genomes has been estimated as lower than $1 \times 10^{-3}$ copies per cell based on the intensity of the hybridization signals. Moreover, the DNA samples extracted from the corresponding non-cancerous esophageal mucosa were also positive for HPV-18, and the intensities of the hybridization signals were almost the same as those of the tumors. The results of our study indicate that HPV-16, HPV-18 and EBV are not generally associated with esophageal carcinogenesis.

Key words: human papillomavirus – Epstein–Barr virus – polymerase chain reaction – esophageal squamous cell carcinoma – esophageal carcinogenesis

INTRODUCTION

The recent interest in human papillomavirus (HPV) and its association with cervical squamous cell carcinoma (SCC) (1–4) has prompted the investigation for its possible etiological role in cancers at other body sites. In lesions of the skin (5), oral cavity (6), tongue (7), larynx (8) and lung (9), the presence of HPV was investigated initially by DNA hybridization techniques and recently by polymerase chain reaction (PCR).

HPV infection of the esophagus was first suggested by morphologic studies showing HPV-induced cytopathic changes (10) and later by immunohistochemical techniques demonstrating the presence of HPV antigens (11,12). The presence of the HPV genome in SCC of the esophagus was demonstrated by DNA hybridization techniques and/or PCR. However, the HPV detection rates vary considerably among different authors, ranging from 0% to 49% (13–22). In Japan, Furihata et al. (13) reported that 33.8% of patients with esophageal SCC were positive for HPV DNA by in situ hybridization and Toh et al. (14) reported a prevalence of 6.7% by PCR. It is important to establish whether HPV DNA is demonstrated in Japanese esophageal carcinoma and whether this virus plays a role in the etiology of the disease. In this study we used the PCR technique to detect HPV-16 and HPV-18 DNAs with primers amplifying a portion of the E6 region. The specific HPV DNA sequences amplified by PCR were subsequently confirmed by Southern blot hybridization with the HPV-specific probes.

The Epstein–Barr virus (EBV) was first identified in 1964 in cultured Burkitt’s lymphoma cells (23) and has since been implicated in a variety of benign and malignant lymphoproliferative diseases. The association of EBV with undifferentiated nasopharyngeal carcinoma has been well documented (24,25). However, it remains controversial whether the nasopharyngeal SCC are also EBV-associated. Recently, the involvement of EBV has been demonstrated in gastric adenocarcinoma of various grades of differentiation without prominent lymphoid infiltration (26,27).
Thus it is of interest to determine whether EBV is involved in carcinoma of the esophagus, which is located between the nasopharynx and stomach. In this study we investigated the presence of EBV in squamous cell carcinoma of the esophagus by PCR.

MATERIALS AND METHODS

EXTRACTION OF DNA FROM SURGICAL SPECIMENS AND CULTURED CELL LINES

The surgically resected specimens of esophageal carcinoma were obtained from the National Cancer Center Hospital at the time of surgery, snap-frozen in liquid nitrogen immediately after resection, and stored at –80°C until DNA extraction. Human esophageal carcinoma cell lines TE1–TE6 and TE8–TE13 were kindly provided by Dr. T. Nishihira (Second Department of Surgery, Faculty of Medicine, Tohoku University). Each cell line was established from SCC and was maintained in RPMI-1640 with 7% fetal bovine serum. Human cervical cancer cell lines SKG-IIIa and HeLa were maintained in Ham’s F12 medium with 10% fetal bovine serum and in Eagle’s minimum essential medium with 10% calf serum respectively. SKG-IIIa cells and HeLa cells contain approximately one copy of HPV-16 DNA and 10–20 copies of HPV-18 sequence per haploid genome respectively (2).

DNA was extracted as previously described (28) from the surgical specimens and cell lines. All the DNA samples were suitable for PCR analysis, because the 170 bp (base pair) fragment of the EXP1 gene, which is a gene identified on chromosome 11q13, about 120kb apart from the HST1 gene (29), was successfully amplified (data not shown). The EXP1 primers are:

P40, 5′-AGCATTTTGCTGCTAGGCTG-3′ and
P9, 5′-TGAAACCTCTCATCCGACATCAG-3′.

PCR reaction was performed in a 50 µl volume with 400 ng of template DNAs, 1x PCR Buffer, 10 mM of each deoxynucleotide triphosphate, 0.5 µg of each primer and 2.5 units of Taq-polymerase. The conditions for PCR analyses were as follows: 94°C for 10 min (1 cycle); 94°C for 1 min, 60°C for 2 min, 72°C for 3 min (30 cycles); 72°C for 10 min (1 cycle). After amplification of PCR, 10 µl (corresponding to 1/5 volume of the reaction mixture) was subjected to gel electrophoresis on a 3.5% NuSieve 3:1 agarose gel and stained with ethidium bromide. DNA was subjected to PCR analysis for HPV-16 and HPV-18. After amplification of PCR, 10 µl (1/10 volume) of the reaction mixture was subjected to gel electrophoresis on a 3.5% NuSieve 3:1 agarose gel and stained with ethidium bromide. DNA was subjected to PCR analysis for HPV-16 and HPV-18. After amplification of PCR, 10 µl (1/10 volume) of the reaction mixture was subjected to gel electrophoresis on a 3.5% NuSieve 3:1 agarose gel and stained with ethidium bromide. DNA was subjected to PCR analysis for HPV-16 and HPV-18.

PCR ANALYSIS FOR EBV DNA

Sequences of EBV primers, from published data (30) are as follows:

SL1, 5′-GGACCTCAAGAAAGGGG-3′;
SL3, 5′-GCTCTCGTGTCCCGCCTCC-3′.

The primers amplify and detect an 80-base pair region of the EBNA1 gene. Each PCR reaction mixture (100 µl) contained 600 ng of template DNAs, 1x PCR Buffer, 10 mM of each deoxynucleotide triphosphate, 0.25 µg of each primer, 1.0 unit of Perfect Match (Stratagene, California) and 3.0 units of Taq-polymerase. The conditions for PCR analyses were as follows: 94°C for 10 min (1 cycle); 95°C for 45 sec, 60°C for 45 sec, 72°C for 60 sec (35 cycles); 72°C for 10 min (1 cycle). All experiments were performed in parallel with pDR 2 (Clontech Laboratories, Inc., California), which is the vector containing the EBV EBNA 1 sequence, as a positive control, and with a negative control (HeLa for HPV-16 and SKG-IIIa for HPV-18).

After amplification of PCR, 10 µl of the reaction mixture was subjected to gel electrophoresis on a 3.5% NuSieve 3:1 agarose gel and stained with ethidium bromide. DNA was subjected to PCR analysis for HPV-16 and HPV-18.
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RESULTS

DETECTION OF HPV DNAs

The PCR products amplified with the two sets of primers specific for HPV-16 or HPV-18 DNAs were observed as a specific band of the expected size (140 bp) in the positive controls, SKG-IIIa for HPV-16 and HeLa for HPV-18 (Fig. 1). No band was detected in DNAs from HeLa and SKG-IIIa cells which were used as negative controls for HPV-16 and HPV-18 DNA, respectively.

Ethidium bromide staining of the gel revealed no detectable band for any of the 41 surgical specimens or the 12 cell lines. Moreover, by Southern blot hybridization of PCR products, no HPV-16 genome was detectable in any of the surgical specimens or the cell lines (data not shown). Although all of the 12 cell lines were negative, three out of 41 surgical specimens were positive for HPV-18 DNA. The intensity of their hybridization signals was weaker than that of 1/50 000 diluted HeLa cell DNA. In the DNA samples extracted from the corresponding non-cancerous portions (odd numbers) of the surgical specimens of the esophagus. The band hybridizing to the HPV-18 probe was visible on lane 4 upon an extended exposure. Lane 14: SKG-IIIa cell DNA, containing HPV-16 DNA but not HPV-18 DNA.

DISCUSSION

Kulski et al. (32) detected HPV DNA in esophageal SCC (four of 10 cases) in Western Australia by filter in situ hybridization. However, Loke et al. (15) were unable to detect HPV genome in 37 cases of esophageal SCC in Hong Kong by slot blot hybridization in situ or in situ hybridization. In France, one of 12 esophageal SCC was positive for HPV-18 DNA by in situ hybridization and three others were positive by dot blot hybridization (16). In the Linxian district of China, the high-incidence area for esophageal carcinoma, 71 (19.6%) of 363 patients with esophageal SCC were shown to have HPV DNA in their cancer cells by in situ hybridization (17). In Japan, 24 (33.8%) of 71 patients with esophageal SCC were positive for HPV DNA by in situ hybridization (13). Of the 24 cases, 10 were HPV-16 and the others were HPV-18.

Among the PCR-based studies for the HPV-DNA detection, 25 out of 51 (49.0%) biopsy specimens in esophageal precancerous lesions and squamous cell carcinomas have been reported as being positive in the Linxian district of China (33). The specific HPV DNA sequences amplified by PCR were subsequently confirmed by Southern blot hybridization with specific HPV probes. Togawa et al. (18) identified HPV DNA in 14% (10/72) of esophageal squamous cell carcinomas from different regions of the world by a radioactive nested PCR/restriction fragment length polymorphism analysis. By contrast, Kiyabu et al. (19) detected neither HPV-16 nor HPV-18 DNA in 13 esophageal carcinomas in North American patients, using PCR with primers which amplify the E6 of both HPV-16 and HPV-18. Smits et al. (20) detected HPV DNA in none of the 61 esophageal squamous cell carcinomas by multiple broad spectrum PCR. Among 14 patients with esophageal SCC in South Africa, 6 (42.9%) were positive for HPV DNA in tumor biopsies by PCR with primers which amplify the L1 gene of a wide range of HPVs (21). Toh et al. (14) in Japan showed by PCR that three (6.7%) of 45 biopsy samples of esophageal SCC contained HPV-16 or HPV-18 DNA. In this study, primers for PCR were firstly consensus primers for

Figure 1. Southern blot hybridization analysis of PCR products for the presence of HPV-18 DNA. Lanes 1, 2 and 3: HeLa cell DNA, containing 10 to 20 copies HPV-18 DNA sequence per haploid genome, was serially diluted with salmon testis DNA. Lane 1, dilution at 1/50; lane 2, 1/5 000; lane 3, 1/50 000. Lanes 4–13, five pairs of the cancerous portions (even numbers) and non-cancerous portions (odd numbers) of the surgical specimens of the esophagus. The band hybridizing to the HPV-18 probe was visible on lane 4 upon an extended exposure. Lane 14: SKG-IIIa cell DNA, containing 10–3 copies of HPV-16 DNA but not HPV-18 DNA.

Figure 2. Sensitivity of detection of HPV DNA. SKG-IIIa DNA (one copy of HPV-16 DNA sequence per haploid genome) was diluted serially with salmon testis DNA. Number indicates number of copies of HPV DNA in the diluted DNA specimens. 10–3 copies of HPV-16 sequence could be detected, while 10–4 copies could not.

DETECTION OF EBV DNA

A specific band of the expected size of 80 bp should be detected in the PCR products amplified with two primers specific for EBV DNA, if EBV EBNA1 gene is present in a DNA sample. No EBV EBNA1 specific DNA could be detected by ethidium bromide staining in any of the 41 surgical specimens or the 12 cell lines (Fig. 3).
the simultaneous amplification of the E6-E7 regions for cancer-associated HPV types and secondarily type-specific primers for the E7 regions. In the latter three reports, confirmation by Southern blot hybridization of PCR products has not been performed.

Marked variations in the prevalence of esophageal HPV infections have been reported by different authors. This difference might be due to the difference in the methods used. We have used PCR analysis coupled with Southern blot hybridization to detect the E6 region of the HPV-16 or HPV-18 genome, with adequate positive and negative control. The sensitivity of our PCR detection system was almost the same as that of the tumor cell.

In the present series, three out of 41 surgical specimens were positive for HPV-18 DNA by Southern blot hybridization of PCR products. However, the intensity of hybridization signals in these cases was weaker than that of 1,500,000 diluted HeLa cell DNA, which carries 10–50 integrated copies of HPV-18 DNA per haploid genome (2), indicating that the number of viral genomes in those samples is probably lower than 10–3 copies per haploid genome by Southern blot hybridization of PCR products.

In the present series, three out of 41 surgical specimens were positive for HPV-18 DNA by Southern blot hybridization of PCR products. However, the intensity of hybridization signals in these cases was weaker than that of 1,500,000 diluted HeLa cell DNA, which carries 10–50 integrated copies of HPV-18 DNA per haploid genome (2), indicating that the number of viral genomes in those samples is probably lower than 10–3 copies per haploid genome. The possibility of the specific presence of the HPV genome in a clonal population of the tumor cells is quite unlikely. Moreover, HPV-18 was also positive in DNA extracted from their normal esophageal epithelium and the intensity of the hybridization signals was almost the same as that of the tumor cell.

Therefore we presume that HPV is present in fewer than one in 103 cells in the cancerous as well as non-cancerous portion of the esophagus of those patients as a fortuitous parasite of the organ.

While there is a report indicating 33.8% positive for HPV DNA by in situ hybridization in Japan (13), this has not been confirmed in other studies. The reason for the discrepancy remains to be elucidated, but it could be due to a difference in the methods employed. It is also possible that there may be a geographic variation in the prevalence of HPV infection in Japan.

Recently, an association of EBV with epithelial malignancies without prominent lymphoid infiltration has been demonstrated, especially in gastric adenocarcinoma (26). Very little information is available yet in the literature with respect to the possible association of EBV with another upper digestive tract cancer, SCC of the esophagus. Niedobitek et al. (25) have demonstrated the absence of detectable EBV-DNA in any of the eight squamous cell NPCs, 26 SCCs of the tonsils and 14 cervical cancers examined. We have demonstrated the absence of detectable EBV-DNA in all of the 41 surgical specimens and 12 cell lines of the SCCs of the esophagus.

Our PCR analyses detected no specific association of HPV or EBV genome with esophageal SCCs. The present results strongly suggest that HPV-16, HPV-18 and EBV do not play any significant role in the development of esophageal carcinomas.

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