DETECTION OF EPSTEIN-BARR VIRUS DNA AND HHV-6 DNA IN TISSUE BIOPSIES FROM PATIENTS WITH NASOPHARYNGEAL CARCINOMA BY POLYMERASE CHAIN REACTION

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Abstract. A total of 34 tissue biopsies were collected from nasopharyngeal carcinoma (NPC) patients and 5 controls with non-NPC. Extracted DNA from tissue biopsies were analyzed for presence of specific gene sequences to EBV type A and type B, and HHV-6 by polymerase chain reaction (PCR). The different sequences of EBV type A and B were parts from the highly divergent forms of the EBV nuclear antigen 2 (EBNA 2). The PCR amplified products for EBNA 2A and EBNA 2B were 115 and 119 base pairs respectively whereas that of HHV-6 DNA was 776 base pairs. The results demonstrated that EBV DNA was detected in 32 of 34 cases (94.1%) : 28 (82.3%) with type A, 2 (5.9%) with type B, and 2 (5.9%) with both types. EBV DNA of type A could be detected in 20% of 5 controls. HHV-6 DNA was in 5 of 34 samples (14.7%) whereas HHV-6 DNA was not detectable in biopsy tissues from controls. The results show that in the NPC patient group, A type of EBV is predominant. Detection of HHV-6 DNA in patients group only might be resulted from reactivation of a latent infection or association with EBV-induction of NPC.

INTRODUCTION

Epstein-Barr virus (EBV) is a human herpesvirus that causes infectious mononucleosis and appears to be responsible for two types of human cancers : nasopharyngeal carcinoma (NPC) and Burkitt’s lymphoma (BL). NPC is confined mostly to south China and Southeast Asia while BL is confined to Africa (Klein, 1973). EBV is divided into two types designated type A (B95-8 isolate) and type B (AG876 or Jijoye isolates). These differences stem partly from the highly divergent forms of the EBV nuclear antigen-2 (EBNA-2) coding region within U2 open reading frame (Adkinger et al, 1985; Dambaugh et al, 1984; Rowe et al, 1989). Type A virus has a world-wide distribution and is regarded as the predominant strain in Western countries. By contrast, type B virus has been found mainly in central Africa and New Guinea (Young et al, 1987; Zimber et al, 1986).

A new human herpesvirus, human herpesvirus-6 (HHV-6), was recently isolated from patients with lymphocytic disorders and acquired immune deficiency syndrome (AIDS) (Downing et al, 1987; Salahuddin et al, 1986; Tedder et al, 1987). Yamanishi et al (1988) have presented evidence that HHV-6 is the causative agent of exanthem subitum. In addition, HHV-6 can play a potential role in neurological disorders (Komaroff et al, 1988). Several interesting studies have shown the interaction of HHV-6 and EBV. Several B cell lines infected with EBV were refractory to infection by HHV-6 (Ablashi et al, 1988a; Ablashi et al, 1988b). It was hypothesized that infection by EBV may lead to induction of a receptor for HHV-6 on the B cells. There were some simultaneous rises in serum antibody titers to HHV-6 and EBV (Kirchesch et al, 1988). The possible co-tumorigenic role of HHV-6 has been suggested by studies in the NIH-3T3 mouse fibroblast system (Razzaque, 1990). HHV-6 is a possible cofactor in NPC because of its almost universal prevalence, lymphotropism and involvement of salivary glands. HHV-6 could replicate (Harnett et al, 1990) and hibernate in a latent form (Krueger et al, 1990) in epithelial cells of salivary glands, as does EBV.

Polymerase chain reaction (PCR) is a technique for the amplification of DNA in vitro (Harnett et al, 1990; Komaroff et al, 1988; Yamanishi et al, 1988) of infectious agents that are present in small numbers in clinical samples. The objective of the study was to detect DNA of EBV type A and B,
and HHV-6 by PCR in biopsy tissues from NPC patients and their controls.

MATERIALS AND METHODS

Subjects

Subjects comprised 34 patients with histologically proved NPC and 5 controls with non-NPC. NPC patients included 1 case of WHO type I, 14 cases of WHO type 2, 17 cases of WHO type 3, and 2 cases of unclassified type.

Clinical specimens

Tissue biopsies were collected prior to any clinical treatment from patients and controls attending at Department of Otolaryngology, Siriraj Hospital, Bangkok, Thailand. The samples of approximate 30 mg in weight were kept at -70°C until tested.

Preparation of DNA samples

DNA was extracted from tissue biopsies by modifying the procedure described by Chan et al. (1988). The tissues were homogenized and then extracted with phenol-chloroform-isooamyl alcohol and followed by ethanol precipitation. The DNA precipitate was digested with RNase A at 37°C for 30 minutes and proteinase K at 37°C, overnight. The mixture was reextracted with phenol-chloroform-isooamyl alcohol and followed by ethanol precipitation. The DNA precipitate was dissolved in 20 μl of tris-EDTA buffer, pH 8.0 and then kept at -20°C until used.

Primer synthesis

Two pairs of primers, which were specific to EBNA 2A and EBNA 2B regions, were synthesized based on the published DNA sequences of EBV genome (Adldinger et al., 1985; Dambaugh et al., 1984). The sequences of EBNA 2A primers were 5'-AACTTCAACCCACACCATCA-3' and 5'-TTCTGGACACTATCTGGATCAT-3'. The sequences of EBNA 2B primers were 5'-TACTCTCCTCAACCCAGAA-3' and 5'-GGTGTTGATTGATG-3'. The PCR amplified products were 115 and 119 base pairs in length for EBNA 2A and EBNA 2B, respectively.

HHV-6 DNA was amplified using primers derived from the Hashimoto strain with the sequences in which parts of the SalI fragment (approximately 6 kilobase pairs) are located in the region encoding a major capsid protein as previously described (Kondo et al., 1990; Lawrence et al., 1990). The sequences of HHV-6 primers were 5'-GTGTTCATTGACTGAAACCGGT-3' and 5'-TAACATCAATGCGTGCATATCAGT-3'. The HHV-6 product was 776 base pairs in length. These primers synthesized in a DNA synthesizer (Applied Biosystems, USA).

DNA was amplified in a total volume of 50 μl of a reaction mixture containing 5 μl of sample; 50 pM of the primers; 40 mM of deoxyribonucleoside triphosphates (dNTP); buffer (50 mM KCl, 10 mM Tris hydrochloride pH 8.3, 0.01% gelatin, 1.5 mM MgCl2) and 2 units of Taq polymerase (Perkin Elmer Cetus, USA). The amplification was carried out in a DNA thermal cycler (Hybaid, Japan). The cycle for EBV DNA amplification consisted of denaturation at 90°C for 1 minute, annealing at 50°C for 2 minutes and extension at 72°C for 5 minutes. The cycle for HHV-6 DNA amplification was the same as that described above, except that the annealing temperature was 62°C. The samples were subjected to 30 amplification cycles. A second run of 30 cycles was done for HHV-6 DNA.

Analysis of amplified products

The amplified products were detected by direct gel analysis and southern blot hybridization.

For direct gel analysis, 10 μl of the amplified products was loaded on 1% agarose gels and subjected to electrophoresis. A 3% Nusieve agarose gel was added for detection of EBV DNA. The DNA was visualized by UV fluorescence after staining with ethidium bromide. Molecular weight markers were included in each gel.

For southern blot hybridization, the DNA on gels was transferred to nylon filter membrane (Hybond-N+, Amersham, UK) with 0.4 M NaOH for 3 hours. The filter was neutralized with 2 × SSPE (0.3 M NaCl, 20 mM NaH2PO4 pH 7.4, 2 mM disodium EDTA) for a few minutes. The DNA samples were then hybridized for 12 hours with 32P-labeled cloned probe (1 × 108 cpm/ml) in hybridization fluid (6 × SSPE, 3% skim milk, 0.1% SDS). The filter was then washed twice with 2 × SSPE, 0.1% SDS for 10 minutes each at room
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temperature, once for 15 minutes at 65°C, and then twice with 0.2 × SSPE, 0.1% SDS for 20 minutes at 65°C. Bound probes were detected by autoradiography at -70°C for 8 hours with intensifying screens. Probes used to detect amplified products were cloned DNA probes. For the amplified products of EBV DNA, the probes of plasmid pACYC 184, which contains EBNA 2A (1.5-kilobase pairs) and pUC 8, which contains EBNA 2B (0.75-kilobase pairs) were kindly provided by Dr TB Sculley (QIMR, Australia). Cloned DNA probe which contains a part of the SalI fragment (approximate 6 kilobase pairs) as described above was used for HHV-6 DNA detection.

RESULTS

Detection of EBV and HHV-6 DNA by PCR

The oligonucleotides selected as primers for EBNA 2A and EBNA 2B were specific for detection of different genotypes of EBV strains. The PCR amplified products for EBNA 2A and EBNA 2B were 115 and 119 base pairs respectively (Figs 1A, 1B) whereas that for HHV-6 DNA was 776 base pairs (Fig 1C). The results from direct gel analysis and southern blot hybridization for EBV DNA amplified products were comparable. For amplified products of HHV-6, it gave positive signal in southern blot hybridization only.

Detection of EBV DNA in clinical specimens

EBV DNA was detected in 32 (94.1%) of 34 NPC patients: 28 (82.3%) with type A, 2 (5.9%) with type B, and 2 (5.9%) with both types (Table 1).

![Fig 1—Agarose gel analysis and southern blot hybridization of products amplified by PCR with EBNA 2A primers (A), EBNA 2B primers (B) and HHV-6 primers (C). Molecular weight markers (lane 1) for EBV and HHV-6 products were pUC 19-Sau3A1 and -EcoT14I respectively. Negative controls (-) and positive controls (+) are shown respectively. Lanes 2-10 were applied with DNA amplified products of clinical samples.](image-url)
Table 1

Detection of EBV DNA and HHV-6 DNA in biopsy tissues from NPC patients.

<table>
<thead>
<tr>
<th>Histological types</th>
<th>No. of biopsy tissues</th>
<th>No. of biopsy tissues positive for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EBNA 2A</td>
</tr>
<tr>
<td>WHO type 1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>WHO type 2</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>WHO type 3</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>Unclassified</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(82.3%)</td>
</tr>
<tr>
<td>Controls (Non-NPC)</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(20.0%)</td>
</tr>
</tbody>
</table>

Detection of HHV-6 DNA in clinical specimens

HHV-6 DNA was positive in 5 (14.7%) of 34 samples from NPC patients. Detection of HHV-6 DNA was analyzed against classification of histologic sections of NPC according to the World Health Organization (WHO). Five positive samples were found in WHO 2 and 3 (Table 1). HHV-6 DNA was not detectable in biopsy tissues from non-NPC group.

DISCUSSION

In the study, detection of EBV DNA in 94.1% (32/34) of NPC patients was higher than that in 20% of non-NPC controls. The finding has confirmed an association between EBV and NPC performed by serology as reported previously (Hadar et al, 1986; Puthavathana et al, 1991; Wara et al, 1975; Zeng, 1985). The previous reports demonstrated that EBV DNA sequences were detected in 46 (92%) of 50 NPC specimens by PCR (Chang et al, 1990) and all the eighteen NPC biopsies tested were positive for EBV DNA detection using in situ hybridization (Permeen et al, 1990). Detection of EBV DNA found in 20% of 5 controls was similar to 22% in throat washings (Sixbey et al, 1989) and 12% in oropharyngeal cells (Gopal et al, 1990). Unfortunately, the tissue biopsies from the control group were limited. EBV DNA could be detected in tissues from NPC of all histological types in Thailand. Only one case of WHO type 1 was determined in this study since WHO type 1 NPC was rare in Thailand (Lunchanavanich et al, 1988; Puthavathana et al, 1991).

The study demonstrated that EBV DNA was detected in 82.3% with type A, 5.9% with type B and 5.9% with co-infection of type A and B of NPC patients whereas 20% of controls were found to be only type A. NPC patients were infected predominantly with type A and some with type B or both types. Type B of EBV was described previously as a poorly transforming type (Dambaugh et al, 1984), however, several reports have shown that type B is associated with NPC and BL tumors. The subtypes of EBV found in NPC biopsies were 94.3% type A, 3.8% type B and 1.9% coinfection of type A and B (Shu et al, 1991). EBV type B has been found in up to 40% of BL tumors and about 20% in the peripheral blood lymphocytes of healthy adults in BL-endemic areas of Africa (Young et al, 1987; Zimber et al, 1986). There are some studies that have demonstrated type B EBV infection outside Africa. Young et al (1987), for example, detected type B virus in only 3 of 100 spontaneous lymphoblastoid cell lines from Caucasian donors. The findings in Australia by Sculley et al (1988) showed a higher portion of anti-EBNA-2B in AIDS patients and HIV-infected persons than that in healthy controls. Additional studies on lymphoblastoid cell lines from HIV-positive subjects and controls showed that 19% were infected...
with B-type EBV, 69% with A-type, and 12% with both types (Sculley et al, 1990). These results suggested that both types of EBV may exhibit dual tissue tropism as well as that reported by Sixbey et al (1989). They reported that EBV DNA was detected in the throat washings of 34 (22%) of 157 randomly selected donors, 14 (41%) of whom had type B virus, 17 (50%) type A and 3 (9%) both types. Interestingly, preliminary analysis of donors in their study who were shedding both virus types in the oropharynx showed only type A in peripheral blood lymphocytes.

HHV-6 DNA was found in 5 (14.7%) of 34 biopsy tissues from NPC patients while HHV-6 DNA was not detectable in tissues from controls. The results were similar to the 15% in throat washings from NPC and 3% in those from healthy adults (Kido et al, 1990). In contrast, 63, 23 and 12 percent of samples of oropharyngeal cells from healthy adults were positive for HHV-6, EBV and both HHV-6 and EBV DNA, respectively (Gopal et al, 1990). Detection of HHV-6 DNA in only NPC patients suggested that HHV-6 was reactivated from the latent state or that HHV-6 may be a cofactor in EBV-induction of NPC. These findings revealed that the concept of association between HHV-6 and EBV in NPC is possible. The in situ hybridization for determination of HHV-6 and EBV DNA in the same tumor cells may be supportive of a role of HHV-6 in EBV-associated NPC.

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REFERENCES


