Lack of Association between Epstein-Barr Virus and Epithelial Malignancies Developed after Kidney Transplantation

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Purpose: Organ transplant recipients are at high risk of developing malignancies due to immunosuppressive regimens. Unlike post-transplant lymphoproliferative diseases (PTLDs), where Epstein-Barr virus (EBV) plays an etiological role, there are conflicting data regarding the association of EBV with post-transplant epithelial malignancies. In order to clarify the role of EBV in carcinomas that develop after solid-organ transplantation, the presence of EBV infection in the carcinomas of post-kidney transplant patients was examined.

Materials and Methods: The presence of EBV infection in skin carcinoma (PTSC), gastric carcinoma (PTGC) and urothelial carcinoma (PTUC), which developed in the patients under an immune suppression regime following kidney transplantation, was examined. Tumors from the patients without organ transplantation were also used as a comparison in the study. The study group included five nasopharyngeal carcinomas (NPCs), one Hodgkin’s disease (HD), one B-cell non-Hodgkin’s malignant lymphoma (NHL) and one hypopharynx (HPC) tumor.

Results: Immunofluorescence assay and Western blot analysis, using sera from the same patients, confirmed that all of the tested patients were previously infected with EBV. From in situ hybridization, no EBER positive cells were detected in any of the tumor tissues obtained from the three kidney transplant recipients (PTSC, PTGC and PTUC) or in the NHL and HPC tissues. In contrast, all five of the NPC and HD tissues showed strong EBER positivity.

Conclusion: These results suggest that there is a strong association of EBV with NPC and HD as previously reported, while no such strong association of EBV was found with epithelial malignancies that developed after kidney transplantation. (Cancer Research and Treatment 2003; 35:433-439)

Key Words: Epstein-Barr virus, Kidney transplantation, Epithelial malignancy

INTRODUCTION

Epstein-Barr virus (EBV) is a gamma herpesvirus linked to the development of a various human malignancies, including Burkitt’s lymphoma (BL), Hodgkin’s disease (HD), nasopharyngeal carcinoma (NPC), NK/T cell lymphoma, post-transplant lymphoproliferative disease (PTLD), acquired immune deficiency syndrome (AIDS)-related non-Hodgkin’s lymphoma, and some forms of gastric carcinomas (1). Humans are the exclusive natural host for EBV, with about 90% of adults throughout the world being infected (2). The oropharyngeal epithelium is the primary site of infection and is believed to be the location for virus replication. Active lytic replication in the oropharynx ensures the production of new virions for transfer in the saliva to a new host. Circulating B cells passing through this area may also be infected with new virions. After B cell infection, EBV establishes a latent infection. The fine balance between EBV-induced cell expansion and the host’s immune response enables the EBV to persist in the host in a clinically silent form. Immunosuppression disrupts this balance, and provokes the excessive proliferation of EBV infected cells, leading to the development of EBV-associated malignancies. PTLDs often arise in organ transplant recipients receiving immunosuppressive agents or T-cell depleted allogenic bone marrow transplantation (3). The majority of these disorders are of a B cell origin, and caused by the out-growth of EBV-infected B cells. Other forms of EBV-associated B cell lymphomas, in immunocompromised individuals, are found in AIDS patients; diffuse large B-cell lymphoma and AIDS-related BL are also of a B cell origin, and show a clonal EBV infection (4). EBV has been implicated
in the pathogenesis of epithelial neoplasms since its detection in undifferentiated NPC tissues (5,6). EBV is detected globally in the tissues of about 10% of gastric adenocarcinomas (7), and is believed to play an etiological role for these malignancies. Furthermore, EBV has been found in several rare epithelial malignancies, such as thymic carcinoma (8), salivary gland carcinoma (9,10) and hepatocellular carcinoma (HCC) (11). EBV has also shown an association with epithelial hyperplasia in immunocompromised individuals, and appears to cause epithelial hyperplasia, otherwise known as oral hairy leukoplakia, in AIDS patients (12).

Epithelial malignancies are frequently found in immuno-suppressed organ transplant recipients (13). Interestingly, EBV has been detected in squamous cell carcinomas of some patients who received kidney or cardiac transplantations (14,15). However, other investigators have reported that they failed to detect EBV in carcinomas of immuno-suppressed organ transplant patients (16–18). In order to clarify the association between EBV and the epithelial malignancies that develop in immunocompromised transplant recipients, the presence of EBV infection in carcinomas of Korean post-kidney transplant patients was sought.

MATERIALS AND METHODS

1) Cell culture

EBV positive Raji cells, EBV negative BJAB cells, EBV negative Akata cells [Akata (EBV-)], EBV positive Akata cells [Akata (EBV+)] and a lymphoblastoid cell line (LCL1) were used as the cell lines in this study. All the cell lines were maintained in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, San Diego, CA), penicillin-streptomycin (10,000 U/ml), and fungizone (10,000μg/ml) (Gibco BRL), in a 5% CO2-95% air humidified atmosphere at 37°C.

2) Patients and samples

Among 15 post-kidney transplant malignancies that occurred between Dec. 2000 and to 2001 at Kangnam St. Mary’s hospital, three epithelial carcinomas, with different primary sites, were selected for this study. These malignancies developed between 2 to 13 years after kidney transplantation. Tumor tissues and sera were obtained from these patients: one post-transplant skin carcinoma (PTSC), one post-transplant gastric carcinoma (PTGC) and one post-transplant urothelial carcinoma (PTUC) patients. For comparison, tumors from the patients without organ transplantation were also used in the study. These included five nasopharyngeal carcinomas (NPCs), one Hodgkin’s disease (HD), one B-cell non-Hodgkin’s malignant lymphoma (NHL) and one lymphopharynx (HPC) tumor. Informed consents were obtained from all participants in this study. Histological specimens were fixed in 10% formaldehyde and processed for paraffin embedding. The patients ranged from 24 to 78 years of age. The NPC specimens were subclassified into keratinizing squamous cell carcinomas (WHO-I), nonkeratinizing carcinomas (WHO-II) and undifferentiated carcinomas (WHO-III), according to the WHO classification. Of the NPC specimens used in this study, two were WHO-II and three were WHO-III. Three of the carcinoma patients had received renal transplants, and varying schedules of immunosuppressive drugs, including cyclosporine and azathioprine (Table 1). All the other tumor patients were neither organ recipients nor under immune suppression.

Table 1. Patients’ information

<table>
<thead>
<tr>
<th>No</th>
<th>Age/Sex</th>
<th>Diagnosis</th>
<th>Histological type</th>
<th>Biopsy site</th>
<th>Transplant type</th>
<th>Imm. Supp.*</th>
<th>TNM†</th>
<th>Stage</th>
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<tbody>
<tr>
<td>1</td>
<td>55/F</td>
<td>PTSC</td>
<td>Poorly differentiated adenocarcinoma</td>
<td>Popliteal area</td>
<td>Kidney</td>
<td>CSA4 + +</td>
<td>T2N1M0</td>
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<tr>
<td>2</td>
<td>64/F</td>
<td>PTGC</td>
<td>Poorly differentiated adenocarcinoma</td>
<td>Stomach</td>
<td>Kidney</td>
<td>CSA</td>
<td>T2N1M0</td>
<td>II</td>
</tr>
<tr>
<td>3</td>
<td>47/F</td>
<td>PTUC**</td>
<td>Urothelial carcinoma</td>
<td>Urinary bladder</td>
<td>Kidney</td>
<td>CSA AZT† + +</td>
<td>T2N0M0</td>
<td>II</td>
</tr>
<tr>
<td>4</td>
<td>30/F</td>
<td>HD† † †</td>
<td>HD, nodular sclerosis</td>
<td>SCLN‡</td>
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<td></td>
</tr>
<tr>
<td>5</td>
<td>62/F</td>
<td>NHL† † †</td>
<td>B-cell, small lymphocytic</td>
<td>Neck LN***</td>
<td></td>
<td></td>
<td></td>
<td>IV</td>
</tr>
<tr>
<td>6</td>
<td>78/M</td>
<td>HPC†††</td>
<td>Moderately differentiated squamous cell carcinoma</td>
<td>Hypopharynx</td>
<td></td>
<td>T4N0M0</td>
<td>IVA</td>
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</tr>
<tr>
<td>7</td>
<td>67/M</td>
<td>NPC 1</td>
<td>Non-keratinizing carcinoma</td>
<td>Nasopharynx</td>
<td></td>
<td>T3N1M0</td>
<td>III</td>
<td></td>
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<tr>
<td>8</td>
<td>24/F</td>
<td>NPC 2</td>
<td>Undifferentiated carcinoma</td>
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<td>T4N1M0</td>
<td>IVA</td>
<td></td>
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<tr>
<td>9</td>
<td>41/M</td>
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<td>Mod. diff. squamous cell carcinoma</td>
<td>Nasopharynx</td>
<td></td>
<td>T4N0M0</td>
<td>IVA</td>
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<td>10</td>
<td>77/M</td>
<td>NPC 4</td>
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<td>T2N2Mx</td>
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<td>11</td>
<td>43/M</td>
<td>NPC 5</td>
<td>Non-keratinizing carcinoma</td>
<td>Nasopharynx</td>
<td></td>
<td>T1N1M0</td>
<td>IIB</td>
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*immunosuppression, † † three significant events in the life history of a cancer [T: the extent of the primary tumor (T1, T2, T3, and T4), N: the extent of regional lymph node metastasis (N0, N1, N2, and N3), M: the absence or presence of distant metastasis (M0, and M1)], female, † male, † † post-transplant skin carcinoma, † † † post-transplant gastric carcinoma, **post-transplant urothelial carcinoma, † † † Hodgkin’s disease, † † † non-Hodgkin’s lymphoma, † Hypopharynx carcinoma, † † nasopharynx carcinoma, † † Supraclavicular lymph node, † † † neck lymph node, † † † † Cyclosporin A, and † † † † † Azathioprine
Table 2. Anti-EBV responses measured by IFA and Western blot

<table>
<thead>
<tr>
<th>No</th>
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<th>Western blot</th>
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<tr>
<td></td>
<td></td>
<td>IgA</td>
<td>IgM</td>
</tr>
<tr>
<td>1</td>
<td>PTSC</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>2</td>
<td>PTGC</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>3</td>
<td>PTUC</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>HD</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>5</td>
<td>NHL</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>HPC</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>NPC 1</td>
<td>+</td>
<td>+</td>
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<td>-</td>
</tr>
<tr>
<td>11</td>
<td>NPC 5</td>
<td>+</td>
<td>-</td>
</tr>
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</table>

*Viral capsid antigen, †early antigen-diffuse & restricted, ‡EBV nuclear antigen 1, §EBV nuclear antigen 2, †EBV latent membrane protein 1, *hot tested*

3) Immunofluorescence assay (IFA)

The EBV serological tests were carried out using standard IFA techniques employing the Bion EBV antibody test system (Bion, Park Ridge, IL). The IgA/IgM/IgG type antibodies, for viral capsid antigen (VCA) and early antigen-diffuse & restricted (EA-DR), were detected by following the manufacturer’s protocol.

4) Western blot analysis

Cell lysates (50μg/lane) of Akata (EBV-), Akata (EBV+), and LCL1 were subjected to 8% SDS-PAGE separation, and then transferred to nitrocellulose membranes by electroblotting. The blots were blocked for 1 hr in Tris-buffered saline (TBS), containing 5% low-fat milk, followed by incubation with either a primary antibody or serum. After washing, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, for 2 hr, at room temperature. The blots were washed in TBS, containing 0.1% Tween 20, and the protein bands were visualized using an enhanced chemiluminescence kit from Amersham Biosciences AB (Uppsala, Sweden). The monoclonal antibody PE specific to EBNA-2 was obtained from Novocastra Laboratory Ltd (New Castle, UK). The monoclonal antibody CS1-4 recognizing LMP-1 was purchased from Dakopatts (Glostrup, Denmark). One defined control serum, which selectively detects EBNA-1, was kindly provided by Dr. W-K Lee (Myungji University).

5) In situ hybridization (ISH)

In situ hybridization was performed, using a DIG-AP-Rembrandt kit (Pan Path, Amsterdam, Netherlands), to detect the presence of EBV-encoded RNAs (EBERs). All the reagents and solutions were prepared with diethylpyrocarbonate (DEPC)-treated water. Five μm thick sections were cut from paraffin-embedded tumor tissues. The sections were deparaffinized, dehydrated, predigested and hybridized with DIG-labeled EBER-specific probe. Detection was accomplished with an alkaline phosphatase (AP)-conjugated anti-DIG antibody and NBT/BCIP (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium). The color development was examined every 5 minutes by light microscopy. The slides were washed and counterstained with nuclear fast red before detection. A positive signal was identified by dark blue staining on a pale pink stained background. The Raji cell line, containing 50–100 EBV genome copies per cell, was used as a positive control. As a negative control, the EBV negative Burkitt’s lymphoma cell line, BJAB, was used. Before being processed for the ISH, the Raji and BJAB cells

![Fig. 1. Latent EBV gene expression in the cell lines. Cell lysates of Akata (EBV-), Akata (EBV+) and LCL1 cells were separated on an 8% acrylamide resolving gel. Western Blot analysis was performed using an EBNA1 specific human serum, an EBNA2 specific monoclonal antibody, PE2, or a LMP1 specific monoclonal antibody, CS1-4.](image-url)
were cytocentrifuged on 3-aminopropyl-triethoxysilane-coated slides (Dakopatts, Glostrup, Denmark) and fixed in 4% paraformaldehyde.

RESULTS

1) Testing human sera for antibodies against VCA and EA-DR by IFA

To study the EBV-specific antibody reactivity in the patient’s sera, IFA for the antibodies to EBV capsid antigen (VCA), as well as to the diffuse (D) and restricted (R) components of the EBV-induced early antigen complex (EA-DR), was carried out. As shown in Table 2, seven of the tested patients had antibodies to EBNA-I. The same patients also had antibodies to VCA and EA-DR of the IgG isotype, but not the IgM isotype. Two of the NPC patients (NPC1 and NPC5) had IgA antibodies to VCA and EA-DR.

2) Testing human sera for antibodies against EBNA1, EBNA2, and LMP1 by Western blot

The results obtained with the IFA were confirmed, and then extended by Western blot analysis (Fig. 1). Whole cell extracts of the well characterized EBV positive and negative cells served as antigens for the Western blotting. The EBNA-I specific control serum detected the EBNA-I proteins of a similar molecular weight in latency I Akata (EBV+) and latency III LCL1 cells. As expected, LMP1 and EBNA2 proteins were detected only in the latency III LCL1 cells, using specific monoclonal antibodies CS1-4 and PE2, respectively. In the Akata (EBV-) cells no viral antigens were detected with any of the three antibodies (Fig. 1). The results obtained using 11 sera, at 1:10−1:20 dilutions, are shown in Fig. 2 and Table 2. Each serum was screened against the two EBV-positive cells, Akata (EBV+) and LCL1, in addition to the EBV-negative Akata (EBV-) cells. Antibodies to EBNA1 were detected in

Fig. 2. Detection of antibodies to EBV antigens in sera using Western Blot analysis. Cell lysates of Akata (EBV-), Akata (EBV+) and LCL1 cell lines were separated on an 8% acrylamide resolving gel. Western Blot analyses were performed using sera from various tumor patients at 1:10−1:20 dilutions.

Fig. 3. In situ hybridization of EBER in the cell lines. EBV positive Raji cells and EBV negative BJAB cells were stained with a DIG-labeled EBER oligonucleotide probe.
all the sera, while those to EBNA2 were detected in 6 of the 11 sera (PTSC, NPC4, NPC5, HD, NHL and HPC). However, antibody to LMP1 was not detected in any of the sera, with the exception of the PHC serum, where weak LMP1 specific reactivity was observed. These results suggest that all the 11 patients were previously infected with EBV at the time of blood sampling. However, there were no noticeable serological differences between the organ transplant recipients and the other tumor patients.

3) Detection of EBERs in tumor tissues by ISH

To investigate the presence and localization of the EBV infection, at a single-cell level, ISH of the EBV-encoded nuclear transcripts, EBERs, were carried out. The EBERs specific

![Image of tumor tissue sections](attachment:figure4.png)

**Fig. 4.** In situ hybridization of EBER in the tumors. Sections from the tumor tissues were stained with a DIG-labeled EBER oligonucleotide probe. Original magnification 100×.
oligonucleotide probe was first tested for Raji and BJAB cell lines. With the digoxigenin-labeled probe, EBER positivity was seen in the formalin-fixed Raji cells attached on slides (Fig. 3). Strongly positive cells showed dense nuclear staining. In contrast, the EBV negative BJAB showed no reactivity with the EBERs probe (Fig. 3). In addition, the Raji cells were not stained when the negative control probe was used confirming specificity of the staining (data not shown). No EBERs expression was detected in tissues from the three patients (PTSC, PTGC and PTUC) receiving immunosuppressive therapy, or in the tissues from two other tumor patients (NHL and HPC) (Fig. 4). In contrast, nuclear staining for EBERs was uniformly present in virtually all the neoplastic cells of each of the five NPC cases tested. Hodgkin’s disease tissue also demonstrated nuclear staining, which was most intense in the Reed-Sternberg cells.

**DISCUSSION**

Information from the Cincinnati Transplant Tumor Registry (CTTR) shows that the overall incidence of malignancy after organ transplantation is three to five times higher than in the general population (19). Failing to eliminate malignant transformed cells due to immunosuppression has been thought to cause malignancies after transplantation (20). One additional contributor may be infections with potentially oncogenic viruses. EBV is considered to play a major role in the initiation and progression of various malignancies. EBV is strongly implicated in lymphomas of immunosuppressed patients following organ transplantation. More than 90% of PTLDs are EBV positive (21). EBV has also been identified in squamous cell carcinomas in organ transplant recipients (14,15). In order to examine whether EBV plays any role in the pathogenesis of epithelial carcinomas that developed after kidney transplantation, EBERISH was carried out. EBERs (EBER1 and 2) are small, nonpolyadenylated RNAs, which are actively transcribed up to $10^3 - 10^5$ copies per cell in latently infected cells (22,23). The high abundance of EBERs in EBV infected cells makes their detection a standard approach for the identification of EBV-associated malignancies. The absence of EBERs positive neoplastic cells in all three carcinoma patients that received kidney transplantation suggests that these neoplastic epithelial cells were free of EBV infection. Other viral infections, and/or noninfectious mechanisms, might play a role in the pathogenesis of these carcinomas. For examples, human papillomavirus infections are frequently found, and demonstrated, in the malignant and premalignant skin lesions of immunosuppressed organ transplant recipients (24). Furthermore, some immunosuppressive agents, such as azathioprine, cyclophosphamide and cyclosporine, may directly damage DNA, or aggravate the DNA damage induced by other carcinogens (25).

**CONCLUSIONS**

All three carcinomas from the recipients of kidney transplant were found to be negative for the expression of EBERs, while all five of the NPC tissues, included as controls, were positive. Our findings suggest that EBV is not likely to play a major role in the carcinogenesis of immunocompromised kidney transplant recipients.

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