No Direct Role for Epstein-Barr Virus in American Hepatocellular Carcinoma

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Epstein-Barr virus (EBV) was recently linked to hepatocellular carcinogenesis in Japanese patients. It is not clear whether EBV infection is also associated with hepatocellular carcinoma (HCC) occurring in American patients. We studied 41 cases of HCC from the Los Angeles area for evidence of EBV infection by *in situ* hybridization, immunohistochemistry, and polymerase chain reaction methods. Of 41 cases, 16 were seropositive for hepatitis B virus surface antigen (39%), 9 of 29 tested were seropositive for hepatitis C virus antibody (31%); in total, 22 cases were seropositive for hepatitis B virus and/or hepatitis C virus (53%). Of 41 cases, 1 was positive for EBV-encoded small nonpolyadenylated RNA (EBER)-1 (2%) by *in situ* hybridization. By immunohistochemistry, two cases were positive for EBV nuclear antigen (EBNA)-1 (5%), one was positive for the transactivating immediate early BZLF1 (ZEBRA) (2%), and none was positive for latent membrane protein-1. None of the 41 cases was positive for latent membrane protein-1 and EBV nuclear antigen (EBNA)-4 DNAs by polymerase chain reaction assay. All four positive cases showed rare EBER-1-, ZEBRA-, or EBNA-1- positive cells (<0.1%); in none of these cases was there expression of any other EBV viral genes. In the one case each that was positive for EBER-1 and ZEBRA, both of which occurred in patients of non-Asian ethnicity, the staining was limited to infiltrating small lymphocytes, and tumor cells were negative. In the two cases that were positive for EBNA-1, both of which occurred in patients of Asian ethnicity, the staining was limited to tumor cells, and infiltrating small lymphocytes were negative. Our study indicates that rare cases of American HCC may contain EBV-infected cells, but it is unlikely that EBV plays a major role in the carcinogenesis of HCC. (Am J Pathol 2001, 159:1287-1292)

and gastric carcinoma.⁶ EBV infection in these malignancies can be demonstrated through the detection of a variety of different EBV gene products by immunohistochemical or molecular assays. The EBV gene expression pattern in a tumor depends on the status of the infected cells (latent *versus* mixed latent and lytic). In the latent cycle, EBV-infected cells usually show three major EBV gene expression patterns, termed latency I, II, and III. In latency I, the infected cells express the EBV-encoded small nonpolyadenylated RNAs (EBERs) and EBV nuclear antigen (EBNA)-1. In latency II, the infected cells express EBNA-1, EBERs, and latent membrane proteins (LMPs). The infected cells essentially express all 10 EBV latent genes in latency III. All three forms of latency can be induced directly into lytic cycle with the activation of the transactivating immediate early BZLF1 (ZEBRA) and BRLF1 proteins. Therefore, EBV-infected cells in lytic cycle express ZEBRA protein.

The importance of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection in the development of hepatocellular carcinoma (HCC) has been well established by epidemiological and molecular studies.^{7,8} Epidemiological studies have also shown that EBV infection often overlaps with HBV and HCV infections where the incidence of HCC is high, such as in Africa, Japan, and Taiwan. Sugawara and colleagues⁹ recently demonstrated that EBV DNA could be detected in 37% of Japanese HCC patients by Southern blot hybridization. In a second study, EBV DNA was detected in 33% of cases of HCV-associated HCC in Japanese patients by polymerase chain reaction (PCR) assay.¹⁰ These results suggest that EBV may play a role in the carcinogenesis of HCC.

The incidence of EBV infection in American HCC patients has not been studied. We investigated EBV expression in 41 HCC patients from the Los Angeles area, studying EBV viral proteins (LMP-1, EBNA-1, ZEBRA) by immunohistochemistry, EBV viral RNA (EBER-1) by *in situ* hybridization, and the presence of EBV viral DNA (LMP-1 and EBNA-4) by PCR assay.

Epstein-Barr virus (EBV) has been associated with several human malignancies, including classical Hodgkin's lymphoma,^{1,2} Burkitt's lymphoma,³ nasopharyngeal carcinoma,⁴ immune deficiency-associated or posttransplantation-associated lymphoproliferative disorders,⁵

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Materials and Methods

Patients and Tissue Samples

Cases of HCC (primary and metastatic) were found in the surgical pathology file at the Department of Pathology at City of Hope National Medical Center. Forty-one cases were selected from the years 1974 to 1999. The tissues had been routinely fixed in 10% neutral formalin and embedded in paraffin. One paraffin tissue block with tumor was selected from each case. The cases were also examined for unusual number of lymphocytes (including plasmacytoid lymphocytes), which are defined as clusters or sheets of small lymphoid cells accounting for $\geq 10\%$ of tumor volume, either within the tumor or at the infiltrating edges. The clinical data and hepatitis serum testing results were abstracted from the medical record. The serum HBV antigen test was performed in all 41 patients, whereas the serum HCV antibody test was performed in 29 patients after 1990.

EBER-1 in Situ Hybridization

The *in situ* hybridization study methods have been previously described.¹¹ Briefly, we used a probe from a region of the EBV genome that is actively transcribed in latently infected cells, a 30-base oligonucleotide complementary to a portion (bp 69 to 98) of the EBER-1 gene. The sequence was 5'-AGA CAC CGT CCT CAC CAC CCG GGA CTT GTA-3' (Operon Technologies, San Pablo, CA). The probe was labeled with biotin at its 3' end. Paraffin sections were deparaffinized and digested with pronase (nuclease-free). Sections were incubated with prehybridization solution and then hybridized with sheared salmon sperm and yeast tRNA along with the appropriate amount of probe. The probe was used at a concentration of 0.25 ng/ μ l with overnight hybridization. Sections were then incubated in a solution of avidin-alkaline phosphatase conjugate, washed for 3 minutes, incubated in McGadey's substrate, briefly washed in distilled water, air-dried, and coverslipped. No counterstain was used. A poly d(T) was used as a control for total RNA preservation, and a known EBV-positive case of nasopharyngeal carcinoma was used as a positive control. A case was considered positive if the nucleus, or nucleus and cytoplasm, of a tumor cell stained dark blue or black.

EBNA-1, LMP-1, and ZEBRA

Immunohistochemistry

Dr. Grasser (Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene und Institut für Pathologie, Universitätsklinik des Saarlandes, Homburg, Germany) kindly provided the rat monoclonal antibody clone 2B4 to EBV EBNA-1.¹² We also used two other antibodies, mouse monoclonal antibody clone CS1-4 to LMP-1 protein (DAKO, Carpinteria, CA) and clone BZ.1 to ZEBRA (BamHI Z fragment, Epstein-Barr-replication activator) protein (DAKO). EBNA-1, LMP-1, and ZEBRA immunohistochemistry were performed in all 41 cases of

HCC. Paraffin sections were deparaffinized and rehydrated in a graded alcohol series. Two of the antibodies required heat-induced epitope retrieval using 100 mmol/L of ethylenediaminetetraacetic acid buffer (pH 8.0) or 10 mmol/L citrate buffer (pH 6.0), for EBNA-1 and ZEBRA, respectively, in a steamer (Black and Decker, Shelton, CT) at 100°C for 20 minutes. The sections were then incubated with 2B4 at 1:500 dilution at room temperature overnight, with CS1-4 at 1:320, or with BZ.1 at 1:20 dilution at room temperature for 40 minutes and washed three times (5 minutes each) with phosphate-buffered saline (PBS) buffer. The sections were then incubated with a biotinylated goat, anti-rat antibody (Vector Laboratories, Burlingame, CA) (for EBNA-1) at a dilution of 1:150, or biotinylated goat, anti-mouse/anti-rabbit antibody (Ventana Medical Systems, Tucson, AZ) (for LMP-1 and ZEBRA) at a dilution of 1:8, followed by application of two washes (5 minutes each) of PBS buffer, followed by avidin-biotin complex (Vector). The slides were counterstained with hematoxylin. Sections of known EBV-positive classical Hodgkin's disease were used as positive controls for EBNA-1 and LMP-1, and tissue sections of infectious mononucleosis were used as a positive control for ZEBRA. Positive staining was interpreted as nuclear or granular nuclear (EBNA-1), membrane and cytoplasmic (LMP-1), or nuclear (ZEBRA) in the tumor cells.

PCR Studies for EBNA-4 and EBV-LMP-1

Viral genomic DNA was extracted from formalin-fixed, paraffin-embedded tumor tissues, using 0.2 mg/ml of proteinase K digestion buffer overnight, followed by denaturation by boiling. The PCR studies were performed with 2 μ l of extracted DNA in a 30- μ l mixture containing 50 mmol/L KCl, 10 mmol/L Tris buffer, pH 8.3, 50 μ M of each deoxynucleotide triphosphate, 2.5 mmol/L MgCl₂, 1 U of *Taq* polymerase (Perkin Elmer, Foster City, CA), and 20 pmol of each primer. We used primers for EBNA-4 that flank the DNA region coding for epitopes of 399 to 408 and 416 to 424 of the prototype B95.8 EBV virus, using the nucleotide positions 96541 to 96540 and nucleotide positions 96770 to 96751 (EBV GenBank Accession Number V01555), respectively: EBNA-4 + 5'-GAG GAG GAA GAC AAG AGT GG-3' and EBNA-4-5'-GAT TCA GGC GTG GCT CTT GG-3'. The expected EBNA-1 PCR product size was 230 bp. We also used primers for the EBV-LMP-1 gene that flank the site of the characteristic 30-bp deletion of LMP-1 gene, using the nucleotide positions 168350 to 168331 and nucleotide positions 168190 to 168209 (EBV GenBank Accession Number V01555), respectively: LMP-1 + 5'-CGG AAG AGG TTG AAA ACA AA-3' and LMP-2-5'-GTG GGG GTC GTC ATC ATC TC-3'. The expected LMP-1 gene product size was 161 bp. After initial denaturation for 5 minutes at 95°C, 45 amplification cycles were performed as follows: denaturing at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 40 seconds. A final extension at 72°C for 7 minutes completed the PCR amplification. The PCR setup and the work after PCR were

Table 1. Summary of Four EBV-Positive Hepatocellular Carcinomas

Case no.	Age	Sex	Ethnic groups	HBV	HCV	<i>In situ</i> hybridization EBER-1	Immunohistochemistry			PCR	
							EBNA-1	LMP-1	ZEBRA	LMP-1	EBNA-4
1	72	F	Caucasian	—	—	+	—	—	—	—	—
2	49	M	Caucasian	—	—	—	—	—	+	—	—
3	32	M	Asian	+	—	—	+	—	—	—	—
4	59	M	Asian	—	—	—	+	—	—	—	—

performed in separate laboratories to minimize the possibility of contamination. Primers flanking β -globin gene were used as a positive control for DNA preservation (expected PCR product size was 268 bp), whereas a known EBV-positive case of T/NK cell lymphoma was used as a positive control.

Results

Clinical Features

The ages of the patients ranged from 16 to 89 years old with a mean age of 58 years and a median age of 62 years. Sixteen of 41 were Asians (39%) and 25 were Caucasians. One third were women (13 of 41) and two-thirds were men (28 of 41). Paraffin sections of all 41 cases contained invasive HCC; 11 were well differentiated, 21 were moderately differentiated, and 10 were poorly differentiated. Of the 41 cases, 22 were wedge resection or autopsy specimens, and 19 were liver biopsy specimens. For the wedge resection and autopsy specimens, the tumor size ranged from 1.5 cm to 24 cm with a mean size of 9.8 cm.

Incidence of HBV and HCV Infection

Sixteen of 41 cases were seropositive for HBV and 9 of 29 cases (after 1990) were seropositive for HCV. In total, 22 of the 41 cases were seropositive for HBV and/or HCV. Of 22 cases with evidence of HBV and/or HCV infection, only 1 case showed evidence of EBNA-1 positivity (case 3 in Table 1) by immunohistochemistry. Eleven of 22 patients were Asians and 11 were Caucasians. The incidence of HBV and/or HCV infection was higher in Asian-American HCCs (69%, 11 of 16) than in Caucasian-American HCCs (44%, 11 of 25).

In Situ Hybridization for EBER-1

Forty-one cases of HCC were hybridized with poly d(T) and EBER-1. All cases showed strong nuclear positivity for poly d(T) (control for RNA preservation). Of the 41 cases, only 1 case showed nuclear positivity for EBER-1 (Table 1 and Figure 1). In this case, rare nuclei of the infiltrating lymphocytes were positive, whereas the tumor cell nuclei were negative. The EBER-1-positive cells constituted <0.1% of the total cell population.

Immunohistochemical Studies for EBNA-1, ZEBRA, and LMP-1

Two of the 41 cases of HCC were positive for EBNA-1, showing a granular nuclear staining (Table 1 and Figure 2A). Tumor cells, and not lymphocytes, were positive for EBNA-1. The percentage of EBNA-1-positive tumor cells was <0.1% of the total tumor cells in both positive cases. One of the 41 cases showed nuclear positivity for ZEBRA in rare lymphocytes (<0.1%) (Figure 2B). None of the 42 cases was positive for LMP-1.

EBV-LMP-1 and EBNA-4 PCR Studies

DNA from formalin-fixed and paraffin-embedded tissues of all 41 cases were purified and amplified with primers flanking EBV-LMP-1, EBNA-4, and β -globulin (control for DNA preservation). Strong β -globin amplified bands were identified from all 41 cases, indicating that the quality and quantity of the purified DNAs were good. None of the 41 cases showed amplified EBV-LMP-1 or EBNA-4 DNA bands.

Correlation of EBV Gene Expression with Lymphocytic Infiltration

Eleven of the 41 cases (27%) of HCC showed a prominent lymphoplasmacytic infiltrate. In these cases, the majority of the infiltrating lymphocytes were located between tumor nodules. Prominent lymphocytic infiltration was present in the EBER-positive case, the ZEBRA-positive case, and one of two EBNA-1-positive cases.

Co-Expression of EBV Genes in HCC

Of the four (4 of 41) EBV-positive cases, none co-expressed more than one EBV viral gene (Table 1).

EBV Gene Expression in Different Ethnic Groups

The two EBNA-1-positive HCCs both occurred in Asian patients (2 of 16, 12.5%). The ZEBRA-positive HCC and the EBER-1-positive HCC occurred in Caucasians (1 of 25, 4% each).

Figure 1. EBER-1 RNA *in situ* hybridization in a 72-year-old Caucasian female with poorly differentiated HCC (case 1). Scattered infiltrating small lymphocytes show nuclear positivity (**solid arrows**); whereas the carcinoma cells (**top left; open arrow**) are negative. The **inset** shows the nuclear EBER-1 positivity.

Discussion

EBV is a ubiquitous virus, infecting >90% of humans and persisting for the lifetime of the individual.¹³ In normal adults, from 1 to 50 B-lymphocytes per million in the circulation are infected with EBV, and the number of latently infected cells within a person remains relatively stable throughout years.¹⁴ In nonneoplastic lymphoid infiltrates, it has been estimated that ~1 in 1000 to 1 in 10,000 lymphocytes are EBV-positive in EBV-seropositive individuals.¹⁵ Only a small population of latently infected B-lymphocytes enters the lytic cycle, marked by expression of nuclear ZEBRA protein. Case 1 and case 2 (Table 1) may represent such cases in which the EBER-1-positive and ZEBRA-positive cells are infiltrating small lymphocytes; whereas the tumor cells themselves are EBV-negative.

EBNA-1 immunohistochemistry, using the 2B4 monoclonal antibody, gave granular nuclear staining in rare neoplastic cells in two cases of HCC. Neither of these two cases was positive for any other EBV gene products, nor did PCR studies reveal evidence of EBV genomes in the tissues. We cannot rule out the possibility that these signals are nonspecific and not actually reflective of the presence of EBV. The 2B4 monoclonal antibody has shown nonspecific staining in various EBV-negative tissue samples, including normal breast tissue, and in various other epithelia.^{16,17}

In two separate studies, Sugawara and colleagues^{9,10} detected EBV DNA in 37% and 40% of HCCs in Japanese population by PCR (detecting EBV *Bam*HI W sequences) and Southern blot hybridization, respectively. The majority of these Japanese HCCs had evidence of HBV and HCV infection. The incidence of EBV positivity in HCV-positive HCC was found to be much higher than HBV-positive HCCs (by a 10:1 ratio). The authors concluded that the EBV-infected HCCs might use the *Bam*HI Q promoter to transcribe the EBNA-1 gene, but not other EBNA, EBER, LMP, or ZEBRA genes. This novel restricted EBNA-1 latent gene expression in HCC tissues has not yet been reported in other EBV-infected cells or malignancies. Two human tumors that are associated with restricted EBNA-1 expression are Burkitt's lymphoma¹⁸ and gastric carcinoma.¹⁹ However, EBERs are always expressed in these two malignancies. Whether the restricted EBNA-1 expression in HCC is a novel, previously unidentified latency pattern remains to be resolved.

There are several differences in experimental design between the current study and the study by Sugawara and colleagues, including the use of different PCR primers, a different patient population, and the HCV status in HCC.¹⁰ They amplified the *Bam*HI W region of EBV DNA by PCR, whereas in current study, we amplified EBV-LMP-1 and EBNA-4 DNA. The *Bam*HI W region is reiterated 7 to 12 times in the EBV genome, thus providing a

Figure 2. **Left:** Granular nuclear EBNA-1 positivity in carcinoma cells in a 59-year-old American-Japanese man with a well-differentiated HCC (case 2). There are no lymphocytes in this field. **Right:** A ZEBRA-positive small lymphocyte in a 49-year-old Caucasian woman with a moderately differentiated HCC (case 2). The tumor cells (arrows) in this case were negative for ZEBRA protein.

good target for the detection of EBV in a sample in which a small viral copy number might be expected.²⁰ Unlike the *Bam*HI W region, there is only one copy of LMP-1 DNA and one copy of EBNA-4 DNA in EBV genome. However, after 45 cycles of PCR amplification, the differences between amplified *Bam*HI W DNA and amplified LMP-1 and EBNA-4 DNAs may be minimal. We have not had difficulty identifying evidence of EBV infection in other EBV-associated neoplasms using an identical technique.^{4,21-23} Therefore, we do not think that the choice of PCR primers should significantly affect the overall results. Furthermore, EBV DNA was also detected by Southern blot hybridization in one Japanese study, indicating a relatively high level of EBV that should certainly have been detected by PCR if present.

Geographical variation in the frequency of EBV infection has been observed in many EBV-associated neoplasms, including nasopharyngeal carcinoma, Burkitt lymphoma, and Hodgkin's disease. For example, the incidence of EBV infection in African Burkitt lymphoma is much higher than in many other parts of the world, and the incidence of EBV-associated Hodgkin's lymphoma is higher in Latin America than in developed countries.^{22,24} There are few studies directly comparing EBV-associated gastric carcinoma in Japanese *versus* American patients, although EBV involvement has been observed in ~7% of gastric carcinoma in Japan,²⁵ 16% in Los Angeles,²¹ and 10.2% in Japanese American man and women in Ha-

waii.²⁶ Therefore, the limited epidemiological data on EBV infection in Japanese and American gastric carcinomas cannot explain why the EBV infection rate is higher (30 to 40%) in Japanese HCCs than in American HCCs. The highest recorded rate of HCC in America occurs among ethnic Asians (American-Chinese or American-Japanese) in Los Angeles.²⁷ Yet, in the current study, we did not observe an increased EBV infection rate in Asian-American HCCs, nor did we observe difference in the EBV infection rate between Asian-American HCCs and non-Asian-American HCCs from the Los Angeles area. Thus it seems that ethnic background may not play a significant role in the frequency of EBV infection in HCC.

In the study by Sugawara and colleagues,⁹ 31 of 35 cases (80%) of Japanese HCC had HCV and/or HBV infection. In the current study, we observed ~60% of American HCC patients had HBV and/or HCV infection. In addition, the incidence of HBV and/or HCV antigenemia in American patients with HCC is >90%.²⁸ Thus the hepatitis virus status should not have affected the final results of EBV infection in American HCC patients. Pathological studies have shown that >80% of patients with HCC have cirrhosis.²⁹ HCC is unusual in patients with primary chronic viral hepatitis but is common when the cirrhosis is secondary to chronic viral hepatitis.²⁷ Epidemiological studies have suggested that most cases of cirrhosis associated with HCC were caused by infection with HBV

and HCV. Therefore, chronic hepatitis-associated cirrhosis per se seems to predispose to HCC,³⁰ whereas the possibility of direct carcinogenic effects of HBV and HCV are still under study.³¹ EBV EBNA-1 has been proposed to play an indirect role in the carcinogenesis of HCC by enhancing HCV replication.³² Therefore, the co-infection EBV and HCV may contribute to HCC in the Japanese population by inducing hepatic cirrhosis.

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