No Significant Association of Epstein-Barr Virus Infection with Invasive Breast Carcinoma

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We studied 48 cases of invasive breast carcinoma for evidence of Epstein-Barr virus (EBV), which is associated with many human malignancies. *In situ* hybridization studies to detect the presence of EBV-encoded small nonpolyadenylated RNA (EBER)-1 were performed in paraffin sections. Immunohistochemical studies to detect EBV nuclear antigen (EBNA)-1, latent membrane protein (LMP)-1, and the transactivating immediate-early BZLF1 (ZEBRA) protein were also performed in paraffin sections. The presence of EBV genomic DNA was studied by polymerase chain reaction (PCR) amplification using sets of primers flanking the EBNA-4 and the EBV-LMP-1 genes in frozen tissues. Southern blot analysis using a probe flanking the EBV terminal repeat region was then attempted in cases that were PCR-positive. Five of 48 cases (10%) of breast carcinoma showed focal EBER-positive tumor cells. Twelve cases (25%) were positive for EBNA-1 by immunohistochemistry, all but one different from the EBER-positive cases. None of the cases were positive for LMP-1 or ZEBRA protein by immunohistochemistry. PCR studies for EBNA-4 and LMP-1 were each positive in five cases (including three cases in common). However, Southern blot studies successfully performed in all but one of the PCR-positive cases were completely negative. The identification of EBV by any methodology was not correlated with tumor size, grade, or lymph node status. This study demonstrated evidence of EBV infection in tissues involved by invasive breast carcinomas in a significant subset of cases. However, the lack of localization of EBV infection to a significant population of the tumor cells in any case, the negativity by Southern blot hybridization, and the lack of expression of multiple antigens in any case strongly argue against a significant role for EBV in the pathogenesis of breast carcinoma. (Am J Pathol 2001, 159:571–578)

Epstein-Barr virus (EBV) has been linked with a number of human malignancies, including African Burkitt's lymphoma,¹ classical Hodgkin's disease,^{2–4} posttransplant-

and acquired immune deficiency syndrome-associated lymphoproliferative disorders,^{5,6} sinonasal NK/T cell lymphoma,^{6,7} nasopharyngeal carcinoma (NPC),⁸ and gastric carcinoma.⁹

Breast carcinoma is the most common malignant tumor and the leading cause of cancer death in women in Western countries. Although the etiology of breast carcinoma is not completely understood, genetic background and hormonal effects are believed to play important roles in its development. In recent years, researchers have questioned whether EBV may play a role in the development of breast carcinoma. This hypothesis is based on several observations. First, a subtype of breast carcinoma known as medullary carcinoma has lymphoepithelioma-like features with prominent lymphocytic infiltration, and lymphoepithelioma-like carcinomas in other organs (especially from foregut sites) are often positive for EBV.^{8,10} Second, latently EBV-infected mothers shed EBV into breast milk.^{11,12} Third, EBV-associated lymphomas have been described in the breast.¹³ Studies examining an association between breast carcinoma and EBV have yielded disparate results. Some studies have shown between 20 and 50% of breast carcinoma cases with molecular evidence of the EBV genome.^{14–16} However, other studies have found no molecular or immunohistochemical evidence for an association between EBV and the development of breast carcinoma.^{17–20}

In an attempt to resolve this dispute, we studied the incidence of EBV infection in 48 cases of invasive breast carcinoma by immunohistochemistry [using anti-EBNA-1, latent membrane protein (LMP)-1, and ZEBRA monoclonal antibodies], *in situ* hybridization (EBER-1), polymerase chain reaction (PCR) (using primers detecting EBV EBNA-4 and LMP-1), and Southern blot methods (terminal repeat probe).

Materials and Methods

Patients and Tissue Samples

We searched the Tumor Bank of the Department of Anatomical Pathology at City of Hope National Medical Center for cases of invasive breast carcinoma from the years 1985 to 1999. We retrieved all cases for which both

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frozen tissues and paraffin-embedded tissues were available and this numbered 48 cases. Fresh tumor tissues had been snap-frozen at the time of surgery and were kept in a -70°C freezer. Tissues had also been routinely fixed in 10% neutral formalin and embedded in paraffin. One paraffin tissue block with tumor was selected from each case. The breast carcinomas were graded as I, II, and III, based on histological pattern, nuclear pleomorphism, and mitotic rate (Bloom and Richardson Grading System).²¹ The cases were also examined for unusual numbers of lymphocytes (including plasmacytoid lymphocytes), which we defined as clusters or sheets of small lymphoid cells accounting for $\geq 10\%$ of the tumor volume, either within the tumor or at the infiltrating edges. Clinical data were obtained from the medical record.

EBER-1 in Situ Hybridization

The *in situ* hybridization study methods have been previously described.²² 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/ μL 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 NPC 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 and Institut für Pathologie, Universitätsklinik des Saarlandes, Homburg, Germany) kindly provided the rat monoclonal antibody clone 2B4 to EBV EBNA-1.²³ We also used two other antibodies, mouse monoclonal antibody clone CS1-4 to LMP-1 protein (DAKO Corporation, Carpinteria, CA) and clone BZ.1 to ZEBRA (*Bam*HI Z fragment, Epstein-Barr-Replication Activator) protein (DAKO). EBNA-1, LMP-1, and ZEBRA immunohistochemistries were performed in all 48 cases of breast carcinoma. Paraffin sections were deparaffinized and rehydrated in a graded alcohol series. Two of the antibodies required heat-induced epitope retrieval using 100 mmol/L 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 fresh frozen tumor tissues, using 0.2 mg/ml proteinase K digestion buffer overnight, followed by denaturation by boiling. The PCR studies were performed with 2 μL of extracted DNA in a 30- μL mixture containing 50 mmol/L KCl, 10 mmol/L Tris buffer (pH 8.3), 50 μM of each deoxynucleotide triphosphate, 2.5 mmol/L MgCl_2 , 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 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.

Table 1. Histologic Features of EBV-Positive Breast Carcinomas Detected by *in Situ* Hybridization, Immunohistochemistry, PCR, and Southern Blot Studies

Features	Number of cases	Number of cases positive for methods						Southern blot
		<i>In situ</i> hybridization EBER	Immunohistochemistry			PCR		
			EBNA-1	LMP-1	ZEBRA	EBNA4	LMP1	
Age (years)								
<30	2	0	0	0	0	0	0	0
30–39	3	1	1	0	0	1	1	0
40–49	9	1	2	0	0	0	0	0
50–59	11	0	3	0	0	1	2	0
60–69	19	3	6	0	0	3	2	0
70–79	3	0	0	0	0	0	0	0
>80	1	0	0	0	0	0	0	0
Tumor size								
<2 cm	12	2	4	0	0	2	2	0
2–5 cm	27	1	7	0	0	2	2	0
>5 cm	9	2	1	0	0	1	1	0
Histologic types								
Ductal	43	5	12	0	0	4	4	0
Lobular	5	0	0	0	0	1	1	0
Histologic grade								
I	2	0	1	0	0	0	0	0
II	36	2	6	0	0	4	3	0
III	10	3	5	0	0	1	2	0
Axillary lymph nodes								
Nodes not dissected	11	2	4	0	0	1	0	0
Nodes negative	11	1	5	0	0	1	3	0
1–3 positive	6	0	2	0	0	0	0	0
>3 positive	20	2	1	0	0	3	2	0
Total cases	48	5	12	0	0	5	5	0

EBER, EBV-encoded small nonpolyadenylated RNA; EBNA-1, EBV nuclear antigen-1; LMP-1, latent membrane antigen-1; ZEBRA, transactivating immediate-early BZLF-1; PCR, polymerase chain reaction.

Southern Blot

Frozen tissues were cut into 5- μ m thin sections, to which proteinase K was added up to 50 μ g/ml in TE buffer, then incubated at 50°C overnight. Genomic DNA was extracted with phenol for three times and precipitated with ethanol. The genomic DNAs were then cut with *Bam*HI and *Eco*RI restriction enzymes (New England BioRad, Boston, MA). The cut DNA was run in 10% polyacrylamide gel overnight at 34 V, each lane loaded with 16 μ g of DNA. The positive control consisted of 0.2 to 3.3 μ g of DNA extracted from Raji cells. DNA was transferred to nitrocellulose paper with 0.4 N NaOH, blotted with P-32 labeled EBV probes that flanked the EBV terminal repeat. The film (Kodak B10MAX MR; Eastman-Kodak, Rochester, NY) was exposed for up to 8 days.

Statistical Methods

Clinical and pathological measurements were compared between patients whose tumors showed EBV gene expression and those whose tumors did not, using Pearson's chi-square test.

Results

The results are summarized in Tables 1 and 2.

Clinical Features

All of the patients were women. The ages ranged from 24 to 90 years old, with a median age of 59 years. The tumor size ranged from 1.0 cm to 12 cm, with a median size of 3 cm. Forty-three cases were invasive ductal carcinoma and five cases were invasive lobular carcinoma. Two cases were histological grade I, 36 were grade II, and 10 were grade III. Thirty-seven of 48 cases had axillary lymph node dissections, with an average of 23 nodes examined in each case. Of the 37 cases with axillary lymph node dissection, 11 cases had no nodal disease. Six patients had a total of one to three nodes with carcinoma and 20 cases had more than three lymph nodes with metastatic breast carcinoma.

In Situ Hybridization for EBER-1

All 48 cases of breast carcinoma were hybridized with poly d(T) and EBER-1. All 48 cases showed strong nuclear and cytoplasmic positivity for poly d(T) (control for RNA preservation). Five of the 48 (10%) cases showed nuclear and cytoplasmic positivity for EBER-1 (Figure 1A). The positive cells were tumor cells, which were grouped together topographically, but comprised <0.1% of total tumor cell population in all five cases. None of the EBER-1-positive cases contained an unusual number of infiltrating lymphocytes or lymphoplasmacytoid cells.

Table 2. Summary of EBV-Positive Breast Carcinomas

Case no.	Type	Grade	Age	Nodes	EBER-1 ISH	EBNA-1 IHC	LMP-1 IHC	ZEBRA IHC	LMP1 PCR	EBNA-4 PCR	Southern	Lymphs
1	Ductal	II	61	N/A	+	+	—	—	—	+	—	Y
2	Ductal	III	37	0/16	+	—	—	—	+	—	—	Y
3	Ductal	II	44	N/A	+	—	—	—	—	—	—	Y
4	Ductal	II	46	3/35	+	—	—	—	—	—	—	N
5	Ductal	III	66	33/36	+	—	—	—	—	—	—	Y
6	Ductal	II	50	0/34	—	+	—	—	+	—	—	Y
7	Ductal	III	56	0/18	—	+	—	—	+	+	—	N
8	Ductal	II	33	4/18	—	+	—	—	—	—	—	N
9	Ductal	III	46	N/A	—	+	—	—	—	—	—	N
10	Ductal	II	49	3/21	—	+	—	—	—	—	—	N
11	Ductal	II	58	N/A	—	+	—	—	—	—	—	N
12	Ductal	III	61	1/28	—	+	—	—	—	—	—	Y
13	Ductal	I	61	N/A	—	+	—	—	—	—	—	N
14	Ductal	II	62	0/22	—	+	—	—	—	—	—	N
15	Ductal	III	66	0/19	—	+	—	—	—	—	—	N
16	Ductal	II	67	0/25	—	+	—	—	—	—	—	N
17	Lobular	II	62	9/19	—	—	—	—	+	+	—	N
18	Ductal	II	64	4/21	—	—	—	—	+	+	N/A	Y
19	Ductal	II	37	4/17	—	—	—	—	—	+	—	—

N/A; no lymph nodes were dissected or no tumor tissue available for Southern blot.

ISH, *in situ* hybridization; IHC, immunohistochemistry; PCR, polymerase chain reaction; Southern, Southern blots; Lymphs, prominent lymphoplasmacytic infiltrate; N, without prominent lymphocytic infiltration; Y, with prominent lymphocytic infiltration.

None of the cases had EBER-1 signal in nonneoplastic cells.

Immunohistochemical Studies

Twelve of the 48 cases of breast carcinoma were positive for EBNA-1, showing a granular nuclear staining. Although scattered EBNA-1-positive tumor cells were seen in some cases (Figure 1B), the majority of EBNA-1-positive cells were clustered together (Figure 1C). The percentage of positive tumor cells was low—<1% of total

tumor cells in any positive case. EBNA-1 did not stain any lymphocytes, endothelial cells, or stromal cells in any of the cases. None of the breast carcinoma cases stained for LMP-1 and ZEBRA proteins.

EBV-EBNA-4 and LMP-1 PCR Studies

Strong β -globin (control for DNA preservation) amplified bands were identified from the DNA of all 48 cases, indicating that the quality and quantity of the purified DNAs were adequate. Amplified EBNA-4 bands and am-

Figure 1. EBER-1 *in situ* hybridization shows nuclear positivity in scattered tumor cells (A). EBNA-1 immunohistochemistry shows granular nuclear positivity. The positive tumor cells are either scattered (B) or grouped together (C). In both situations, positive tumor cells are <1% of total tumor cell population.

Figure 2. Seven cases of PCR-amplified LMP-1 genomic DNA are shown from **lane 2** to **lane 8**. **Lane 1** represents a known EBV-positive T/NK cell lymphoma. Three LMP-1-positive cases (Table 2) are seen in **lane 2** (case 2), **lane 3** (case 6), and **lane 8** (case 18). Note that case 18 shows a 30-bp deletion.

plified EBV-LMP-1 bands (Figure 2) were each identified from 5 of 48 cases (10%), including three cases that co-expressed EBV-LMP-1 and EBNA-4. An additional two cases each expressed either EBV-LMP-1 or EBNA-4. Only one of the five (20%) EBV-LMP-1-positive cases showed a 30-bp LMP-1 gene deletion.

Southern Blot

DNA from the seven positive PCR EBV-LMP-1 or EBNA-4 cases was extracted for Southern blot studies. Case 7 (Table 2) did not contain enough DNA for Southern blot study. The other six cases showed no detectable bands (Figure 3) even with loading of a much greater quantity (16 μ g) of DNA than was used for the positive control (3.3 μ g) and despite the fact that the film was exposed to autoradiography for up to 8 days. Sufficient DNA was available in three cases to perform dilution studies up to 0.2 μ g of control DNA. Despite a clearly visible band at the most dilute concentration of control (at a sensitivity of $\sim$ 1 copy/cell), the three cases showed no detectable bands (data not shown).

Correlation of EBV Gene Expression with Clinical Features

We identified no statistically significant difference in EBV gene expression based on tumor grade, tumor size, lymph node status, or patient age.

Figure 3. Southern blot analysis of six LMP-1 and EBNA-4 PCR-positive cases. **Lane 1:** 3.3 μ g of DNA purified from Raji cells. **Lanes 2-7:** 16 μ g of DNA from case 1 to case 6 in Table 2. The film was exposed for 8 days.

Correlation of EBV Gene Expression with Lymphocytic Infiltration

Fifteen of the 48 (31%) cases showed prominent lymphoplasmacytic infiltration that was present primarily at the periphery or the infiltrating edges of tumors. Five of seven cases (71%) that were EBV-positive by PCR studies showed prominent lymphocytic infiltration (Table 2). In contrast, 10 of 41 (27%) EBV-negative cases by PCR showed prominent lymphocytic infiltration. This difference was statistically significant ($P = 0.013$).

Co-Expression of EBV Genes in Breast Carcinoma

A total 19 of 48 (39%) cases were found to be positive for EBV by at least one methodology test. However, only 6 of the 19 cases (31%) were positive by more than one EBV marker (Table 2). Only one case (case 1) was positive for both EBNA-1 and EBER-1. In this case, the localization of EBNA-1 and EBER-1 was compared, and we observed different cell populations expressing the EBNA-1 protein and EBER-1.

Discussion

EBV is one of the most comprehensively studied human oncogenic viruses. The most restricted pattern of viral latent gene expression is seen in African Burkitt's lymphoma (latency I: expressing only EBNA-1 and EBERs), whereas the most unrestricted pattern of viral gene expression is seen in cases of posttransplantation-associated lymphoproliferative disorders (latency III: essentially expressing all 10 EBV latent gene products). An intermediate viral gene expression pattern is seen in Hodgkin's disease and NPC (latency II: expressing EBNA-1, EBERs, and LMPs).¹⁰ Regardless of the latency pattern, virtually all EBV-associated malignancies express EBNA-1 and EBERs.

The expression of EBV viral genes in tumor cells can be detected by *in situ* hybridization (viral RNA expression), PCR and Southern blot methods (viral genomic DNA), and immunohistochemistry (viral protein expression). The gold standard for the identification of a significant association of EBV with a given neoplasm has been *in situ* hybridization demonstration of EBERs within all or virtually all of the neoplastic cells.²⁴ This methodology has high sensitivity and high specificity and has proven useful in the study of EBV in lymphoid malignancies as well as carcinomas. Polymerase chain reaction studies are generally of limited use because they may be overly sensitive and detect EBV DNA in host cells. Thus, PCR studies alone do not provide definitive evidence of EBV within neoplastic cells, as one cannot exclude the possibility that EBV within bystander lymphocytes contributes the positive signal. Indirect evidence for an association of EBV with a given neoplasm is provided by a Southern blot study that shows a high quantity of EBV genomes, particularly when terminal repeat probe analysis shows that

the EBV is present within a monoclonal population of cells. Immunohistochemical demonstration of EBNA-1 expression may provide additional evidence that a given tumor is EBV-positive, but there is much less experience with this methodology, which is also less sensitive.¹⁷

Several groups have examined breast carcinoma tissues for evidence of EBV and have reported widely divergent findings. Using EBER *in situ* hybridization, Iezzoni and colleagues⁸ did not detect EBV in 52 cases of infiltrating breast carcinoma with medullary features (lymphoepithelioma-like carcinoma). Chu and colleagues¹⁸ tested breast carcinoma samples in a high-incidence EBV-associated NPC population and found that none of 60 Taiwanese breast carcinoma cases were positive for EBV, using EBER *in situ* hybridization and EBNA-2 and LMP-1 immunohistochemistry. Using EBER-1 *in situ* hybridization, Glaser and colleagues¹⁹ also failed to demonstrate any positive cases in 107 breast carcinoma tissues from the United States. Recently, Brink and colleagues¹⁷ studied 24 cases of European breast carcinoma and used DNA PCR to detect large internal *Bam*HI-A rightward transcripts of the EBV genome and the single-copy gene BNL1, which is also known as the LMP-1 gene. They found 5 of 24 (21%) positive for the former transcript, of which two also tested positive for the latter. No cases were positive only for BNL1 (LMP-1). However, when they applied reverse transcriptase-PCR for transcripts encoding EBNA-1, they found none of the five DNA PCR-positive breast carcinoma samples to have EBNA-1 transcripts. By EBNA-1 immunohistochemistry (using the 2B4 clone), they found one PCR-positive and one PCR-negative breast carcinoma to be positive. EBER-1 and EBER-2 RNA *in situ* hybridization signals were not detected in any of the breast carcinoma cells, but one of the PCR-positive cases had a small number of EBER-positive lymphocytes. They concluded that there was no evidence for an association between breast carcinoma and EBV because transcription of EBNA-1, EBER-1, EBER-2, and *Bam*HI-A transcripts in breast carcinoma samples could not be detected. The EBV positivity detected by DNA PCR methods was thought to be most likely caused by the presence of EBV-infected lymphocytes in the breast carcinoma samples.

In contrast, Labrecque and colleagues¹⁵ found 19 of 91 (21%) British breast cancers to be EBV-positive using DNA PCR to detect EBER-1 and EBER-2. Luqmani and colleagues¹⁶ also found 15 of 28 (42%) British breast cancers to be positive using a PCR method. However, none of their PCR-positive cases were positive using EBER *in situ* hybridization. Using DNA PCR to detect EBER-2 and LMP-2, Bonnet and colleagues¹⁴ found 51 of 100 (51%) French breast carcinoma cases to be EBV-positive. They also used Southern blot analysis to look for the presence of the EBV genome in seven of their breast tumor specimens. The EBV genome was detected in DNA from all seven EBV PCR-positive breast tumors and observation of the signal was independent of any lymphoplasmacytic reaction in the tumor. By EBNA-1 immunohistochemical study with the use of clones 2B4 (paraffin sections) and 1H4 (frozen sections), they tested nine EBV-positive and six EBV-negative breast carcinomas.

All nine EBV PCR-positive breast tumors exhibited granular nuclear positivity, but none of six EBV PCR-negative tumors were positive. No staining was observed in normal breast epithelial cells and lymphocytes. The authors concluded that the EBV-infected cells comprise a large percentage of breast tumor cells and that the positive signal was not dependent on the presence and proportion of lymphocytes. However, EBV EBER *in situ* hybridization did not show any signal in three of three EBV PCR-positive breast tumors or one lymph node with EBV-positive metastasis.¹⁴

In the current study, we found that 19 of 48 (39%) breast carcinoma cases had evidence of EBV, by at least one method. However, only 6 of 48 (12.5%) were positive with more than one assay. We identified EBER-1-positive breast carcinoma cells in 5 of 48 (10%) cases. However, in all five cases, only a small subset (<1 in 1000 cells) of the neoplastic cells were EBV-positive. This is in contrast to other EBV-associated malignancies, such as NPC, gastric carcinoma, and malignant lymphoma (including Hodgkin's disease), for which all or virtually all of the neoplastic cells are positive within a given case. Peripheral T-cell lymphoma represents a notable exception in which a subset of cases have EBER positivity in only a subset of the neoplastic cells. Although one may postulate that there could have been a more widespread infection with eventual elimination of the virus, a more likely explanation is that EBV infects rare tumor cells after neoplastic transformation has occurred. In addition, when latent state infection switches to lytic state infection, EBV-infected cells may be negative for EBERs. Such a switch is mediated by ZEBRA protein. Therefore, lytic EBV-infected cells are frequently positive for ZEBRA protein.^{25,26} However, none of our cases (including 19 cases with discrepant EBV gene expression) showed immunohistochemical positivity for ZEBRA. In addition, the LMP-1 antibody cocktail used in this study contains a mixture of LMP antibodies, at least one of which recognizes a LMP band expressed late in the virus productive cycle and therefore may detect lytic proteins in addition to latent cycle proteins.²⁷ Therefore, we conclude the discrepant expression of EBV gene products in 19 cases of breast carcinoma most likely did not result from lytic state infection. We also considered the remote possibility that EBER-1 *in situ* hybridization may show false-negative results, which has been shown to be extremely low in many studies. A recent report on hepatocellular carcinoma contained three cases that were positive for EBV by Western blotting studies and reverse transcriptase-PCR analysis but were negative for EBV EBER by *in situ* hybridization.²⁸ However, this study needs confirmation with a larger series.

Twelve of 48 (25%) cases were positive by EBNA-1 immunohistochemical studies, including nine cases positive by this modality alone. We used an antibody (2B4) produced against a λ -cro/lacZ-EBNA-1 fusion protein that has been shown to react in all forms of EBV latency.²³ This antibody has been shown to have nearly equal sensitivity in paraffin or frozen section immunohistochemistry, and equal or superior sensitivity to another anti-EBNA-1 antibody termed 1H4.^{14,23} Both the 2B4 and 1H4

antibodies were also used by Bonnet and colleagues,¹⁴ for whom EBNA-1 immunohistochemistry was one of the primary study methodologies. Although the sensitivity of the EBNA-1 antibodies may be high, the lack of confirmatory evidence of EBV by other modalities in the majority of cases studied in the current report suggests that the antibody specificity may not be absolute. In fact, there may be cross-reactivity to antibodies directed against other epitopes of the EBNA-1 protein. Studies have shown that the irregular GGGGCAGGA repeat motifs of the latent infection cycle EBNA-1 gene are interspersed in cellular DNA. Therefore, monoclonal antibodies to the irregular glu and ala repeating peptide motifs of EBNA-1 may cross-react with cell proteins;^{29,30} we have observed 2B4 EBNA-1 nuclear staining in normal hepatocytes (data not shown). We also considered the possibility that our use of an ethylenediaminetetraacetic acid-based buffer for immunohistochemistry antigen retrieval led to increased sensitivity of antigen detection, as has been described by other investigators.³¹ However, our staining conditions were optimized to minimize any background staining, and false-positives or false-negatives were not seen in any of our control tissues. Therefore, at the current time, we do not consider EBNA-1 immunohistochemical positivity as evidence of EBV infection in the absence of any other confirmatory studies.

EBV is the most ubiquitous viral infection with >90% of the adult population worldwide infected by the virus.³² EBV is known to infect 2 to 60 lymphocytes per million blood lymphocytes, leading to a lifetime latent infection. By PCR, healthy EBV-seropositive donors have been shown to have 10⁴ to 10⁶ EBV-positive B-cells.^{33,34} Other studies of normal lymphoid tissues have shown between 1 in 1000 to 1 in 10,000 infected cells. Therefore, when DNA is extracted from fresh tissue for PCR studies, the contaminated B-cell latent EBV DNA is also potentially amplified, which may lead to a positive PCR result. The amount of amplified EBV DNA is dependent on the degree of tissue blood contamination, lymphocytic infiltration, and the viral copy numbers in the EBV-positive B-cells. Seven of our 48 (15%) cases were positive for EBV by PCR studies for LMP-1 and/or EBNA-4. All but one of these cases were also studied by Southern blot studies, which revealed no evidence of EBV, despite dilution studies showing sensitivity of at least one copy of EBV DNA per cell. A prominent lymphocytic infiltration was found in five of the seven cases, suggesting that rare EBV-positive lymphocytes were genesis of the positive PCR results. Sampling considerations, including peripheral blood contamination of surgically removed tissues, may explain why our PCR-positive cases failed to reveal EBER-positive lymphoid cells.³⁵

Geographical variation of EBV infection has been observed in many EBV-associated neoplasms, including NPC, Burkitt lymphoma, and Hodgkin's disease. For example, the incidence of EBV infection in African Burkitt lymphoma is much higher than in other parts of the world and the incidence of EBV-associated Hodgkin's lymphoma is higher in Latin America than in developed countries.^{10,36} The studies claiming an association between EBV and breast carcinoma cells were all from

European populations.¹⁴⁻¹⁶ In contrast, another report from Europe failed to demonstrate an association of EBV with breast carcinoma.¹⁷ Therefore, it is unlikely that geographical variation alone can explain the high incidence of EBV-positive breast carcinomas found in some series.

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