Review

Epstein-Barr Virus and Breast Cancer: State of the Evidence for Viral Carcinogenesis

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Abstract

As the etiology and progression of breast cancer remain incompletely understood, novel routes of disease pathogenesis are important to consider. Viral pathogens have not been much explored, but recent interest has focused on Epstein-Barr virus (EBV). Studies of an association of this ubiquitous herpesvirus with breast cancer have had notably inconsistent results, marked by varying EBV presence (from 0% to 50% of tumors) and the absence of certain viral characteristics found in other EBV-related malignancies. The research has been plagued by the technical challenges of localizing EBV to tumor cells and by a tendency to overlook epidemiological cofactors, shown in all other EBV-related cancers to impact the EBV association. Breast cancer studies to date have used several viral detection methods of varying or uncertain sensitivity and specificity; most have involved small and/or poorly characterized case series and paid insufficient attention to epidemiological cofactors relevant to breast cancer and to EBV-related malignancies. Given these limitations and the established complexity of the connection of EBV with other cancers, a definitive judgment regarding the presence of this virus in breast cancer cannot yet be rendered. Recent advances in laboratory methodologies should help overcome the challenges of EBV detection in breast cancers. Further research is warranted, given the potential for an EBV association to inform not only breast cancer etiology but also early detection, treatment, and prevention.

Introduction

Breast cancer is the most frequently diagnosed malignancy of women in many populations (1, 2). Research into its etiology has focused primarily on reproductive and other factors affecting circulating sex hormones (3–5), and on genetic susceptibility (6–8). However, as identified risk factors are thought to explain only about half of all breast cancer incidence (9, 10), researchers are motivated to consider other routes of disease pathogenesis. Viruses have been implicated in the development of various cancers (11), but they have not been much considered for breast cancer. Identification of a mouse mammary tumor virus supports a viral etiology for breast tumors in animals, but similar germline viral sequences found in humans are not believed to play any direct role in carcinogenesis (12–14).

In 1995, Labrecque et al. (15) reported finding Epstein-Barr virus (EBV), an ubiquitous herpesvirus, in 21% of a series of 91 breast cancers. A relationship of breast cancer and EBV is potentially important: not only could it broaden understanding of breast cancer etiology, but it also has implications for breast cancer treatment (16), early detection (17), and prevention (18). However, findings from subsequent investigations of this association have been widely variable (19–34). In some part, this inconsistency is attributable to two persistent methodological problems: the technical challenges of localizing EBV to tumor cells, and the tendency to disregard the epidemiological perspective, which could help elucidate variability in EBV prevalence across studies (35). For these reasons, as well as the established complexity of the relationship between EBV and other malignancies (36, 37), it is timely and important to review the evidence for its relationship with breast cancer in the context of EBV pathobiology, the epidemiology of EBV-related cancers, and the current laboratory technologies for EBV detection.

EBV Biology

EBV is a γ herpesvirus; its 184-kb DNA genome encodes approximately 100 genes (36). The virus is transmitted through saliva (38), and primary infection is thought to occur in the oral mucosa (39), typically early in life as a subclinical illness. When infection is delayed until later childhood or adolescence, it manifests in 20–75% of people as infectious mononucleosis (40–42). By adulthood, more than 90% of the population has been infected (43). Primary EBV infection has a replicative (lytic) component marked by production of new virions. After the virus is controlled by immune responses, latent infection persists within a subset of B cells, such that healthy individuals...
EBV and Cancer

As the balance of EBV persistence, virion production, and immune control is well evolved, the vast majority of the world’s population tolerates lifelong EBV infection with no adverse health consequences. However, EBV has been linked to the etiology of several cancers, including African Burkitt lymphoma, in which it was initially described (47); Hodgkin, AIDS, and nasal NK/T-cell lymphomas; post-transplant lymphoproliferative disorder; nasopharyngeal carcinoma (NPC); lymphoepithelioma-like squamous cell malignancies; gastric adenocarcinoma; and leiomyosarcoma (35, 37). EBV is believed to play an active role in their development (36) for several reasons. (a) Viral LMP1 acts as a transforming oncogene in rodent fibroblasts (48–50). (b) The virus has been shown to immortalize B-cell lines in vitro. (c) EBV infection can induce lymphomas in some primates and in immunosuppressed persons. (d) In EBV-associated tumors, the viral genome almost always exists as an episome, that is, an inherently unstable extrachromosomal form (51, 52) that could be lost from a proliferating cell population unless it lent a selective growth advantage. (e) Most EBV-related cancers contain a monoclonal form of the EBV genome in every tumor cell (52, 53), which indicates that infection either preceded malignant transformation or conferred an advantage to an already malignant cell and its progeny. (f) Transfection of the p31 subfragment of EBV DNA immortalizes human epithelial cells, including mammary epithelium (54, 55). (g) EBV-specific antibody titers are elevated before diagnosis of Burkitt and Hodgkin lymphoma, NPC, and gastric adenocarcinoma (56–59). (h) In NPC, EBV-specific antibody titers correlate with tumor burden (60).

On the basis of this evidence, EBV was classified as a group-I carcinogen in 1997 (39). However, as shown in Table 1, the associated cancers vary markedly in viral prevalence, from nearly 100% of NPCs to about 10% of gastric carcinomas (35, 44) and also differ in the patterns of viral genes expressed, suggesting that EBV may affect cell growth in more than one way (37). Thus, EBV infection represents an important but not a sufficient step in carcinogenesis, and epidemiological risk factors have been shown to play an additional critical role in this process.

Table 1. Prevalence of EBV and viral gene expression in EBV-related cancers and other diseases (44)

<table>
<thead>
<tr>
<th>EBV-associated disease</th>
<th>Expressed antigens</th>
<th>Latency type</th>
<th>EBV positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>African Burkitt lymphoma</td>
<td>-EBNA-1</td>
<td>I</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>Sporadic Burkitt lymphoma</td>
<td>-EBERs</td>
<td></td>
<td>20%</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>-EBERs</td>
<td></td>
<td>6–16%</td>
</tr>
<tr>
<td>Nasopharyngeal carcinoma, Asian</td>
<td>-EBNA-1</td>
<td>II</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>Nasopharyngeal carcinoma, North American</td>
<td>-LMP-1, -2A, -2B</td>
<td></td>
<td>75%</td>
</tr>
<tr>
<td>Hodgkin lymphoma</td>
<td>-EBERs</td>
<td></td>
<td>40%</td>
</tr>
<tr>
<td>Hodgkin lymphoma, AIDS-related</td>
<td>-EBERs</td>
<td></td>
<td>&gt;90%</td>
</tr>
<tr>
<td>Nasal T/NK-cell lymphomas</td>
<td>-EBNA-1, -2, -3A, -3B, -3C, -LP</td>
<td>III</td>
<td>40%</td>
</tr>
<tr>
<td>AIDS-associated non-Hodgkin lymphoma</td>
<td>-LMP-1, -2A, -2B</td>
<td></td>
<td>95%</td>
</tr>
<tr>
<td>Post-transplant lymphoproliferation</td>
<td>-EBERs</td>
<td></td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Infectious mononucleosis</td>
<td>-EBNA-1, -2</td>
<td>Other</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>Leiomyosarcoma in immunocompromised hosts</td>
<td>-EBERs</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Epidemiological Cofactors in EBV-Associated Malignancy

EBV-related malignancies show considerable geographic and racial/ethnic variation in incidence, implicating regional and environmental cofactors in carcinogenesis. Nevertheless, they share several epidemiological risk factors, as recently reviewed (35). They tend to affect persons both at young ages, suggesting genetic predisposition or important early-life exposures, and at older ages, when diminishing immune control of infection may be a cofactor. Despite similar seropositivity in males and females, many EBV-related cancers are more common in males, possibly due to females’ more active immunological response to EBV. Some EBV-related cancers occur in families, indicating genetic susceptibility, and some are associated with polymorphisms of the human leukocyte antigen complex, involved in immunological regulation of viral infection. Many EBV-related cancers are more common in persons of lower socioeconomic status, and some have been linked with concomitant infections (HIV, Plasmodium falciparum malaria, Helicobacter pylori), perhaps because of associated immunosuppression impairing anti-EBV response, and/or through chronic antigenic stimulation. A few EBV-related cancers are associated with chemical exposures (dietary salt, nitrosamines, formaldehyde, certain plant derivatives). Together, these epidemiological patterns suggest that EBV functions as a carcinogen or else drives cell proliferation predisposing to genetic defects but, in either case, works in consort with genetic and/or environmental cofactors.

EBV and Breast Cancer

Indirect Evidence. Indirect support for an association of EBV with breast cancer comes from observations...
that: (a) EBV is present in breast tissue, where it is detected in breast milk in some women (61); (b) transfection of EBV DNA stimulates growth of human breast milk cells (55); (c) some EBV-associated lymphomas occur in the breast (62–65); (d) breast cancer has epidemiological similarities to young-adult Hodgkin lymphoma (66–68), although evidence for breast cancer implicates timing of primary EBV infection rather than viral oncogenesis (66); (e) EBV has been identified in benign breast tumors in immunosuppressed women (69); and (f) in vitro, breast epithelial cells can be infected by direct contact with EBV-bearing lymphoblastoid cell lines (70).

Serological Evidence. No published studies have looked at classic serological markers of EBV in breast cancer patients. Recently, antibodies to BFRF1, an EBV protein related to viral replication, were not detected in any of 71 breast cancer patients but were found in patients with other EBV-associated malignancies (71).

Tumor-Based Evidence. Most specific evidence for an EBV-breast cancer association has come from identification of EBV genes or gene products within tumors (Table 2). Because these studies have used several laboratory methods, their findings must be evaluated in relation to the respective strengths and weaknesses of these analytical approaches.

PCR. PCR is potentially a highly sensitive and specific method to detect EBV DNA. However, it cannot differentiate EBV in tumor cells from EBV in surrounding lymphocytes, a limitation for studying tumors, like breast cancer, that have lymphocytic infiltrates. Among the 15 studies using PCR to detect EBV in breast tumors, virus was found in 0–66% of specimens. Prevalences were highest when PCR targeted the EBER and the reiterated BamH1W sequence (15, 20, 21, 25, 26, 29, 33), more moderate when PCR targeted LMP1 or EBNA4 (26, 31), and lowest in examinations of EBNA1 (19, 27), indicating the importance of the PCR target on the extent of the association. Xue et al (55) amplified EBV DNA in breast cancer tissues and also used reverse transcriptase (rt) PCR to confirm expression of viral BART (BamH1A rightward transcription), LF3, EBNA1, BARF1, and BZLF1. Positive PCR studies failed to find EBV in most healthy tissues adjacent to the EBV-positive tumors (15, 21, 25, 29), indicating that EBV is likely tumor-specific. One study found that lymphocytic infiltrates in breast cancers were more common in EBV-positive than EBV-negative tumors (71% versus 27%, P = 0.01), but that the infiltrates themselves were not EBV-positive (31).

Southern Blot Hybridization. Southern blot analysis is less sensitive than PCR for detecting viral DNA but permits semi-quantification of viral load. With Southern blot studies, Bonnet et al. (25) detected EBV DNA in 7 of 7 tumors selected from among 51 tumors EBV-positive by PCR, but Chu et al. (31) failed to find positive results in any of seven PCR-positive tumors.

In Situ Hybridization. In situ hybridization (ISH) for EBV permits visualization of the virus or its transcripts and thus differentiates EBV in tumor cells from EBV in surrounding lymphocytes. When targeting EBERs, ISH is and thus differentiates EBV in tumor cells from EBV in lymphocytes. IHC for LMP1 is a widely employed assay that is sensitive but limited by the fact that LMP1 is absent in some otherwise EBV-related tumors (Table 1). For breast cancer, six IHC studies targeting LMP1 had negative results (21–23, 27, 31, 32). IHC targeting EBNA1 is a less well-established assay and, although sensitive, not necessarily specific for EBV due to cross-reactivity with cellular proteins (26, 31). Bonnet et al. (25) found EBNA1 by IHC in all nine breast cancers positive for EBV by PCR, although only in 5–30% of the malignant cells. Brink et al. (26) found EBNA1 in one of five tumors (20%) but interpreted the staining as nonspecific due to absence of the corresponding viral transcripts by rtPCR. Preciado (74) described EBNA1 expression in 37% of 102 breast carcinomas. In two studies of specimens not screened by PCR, EBNA1 was found in 42% of 33 tumors, but in only 5–30% of the malignant cells (33), and in 25% of 48 tumors, with positive cells comprising <1% of the tumor mass but no normal cells staining (31). Two other studies had no positive results (32, 34). Thus, IHC for EBNA1 has found overall EBV prevalences of 0%, 0%, 4%, 25%, 37%, 42%, and 51%. IHC targeting EBNA2, LMP2a, and the BZLF1 viral replication factor yielded negative results (31). Assays using the 2B4-1 antibody against EBNA1 found positive results in both PCR-positive and PCR-negative breast cancers, suggesting lack of specificity for EBV (75); the 1H4 antibody clone may be more specific for EBNA1 (34, 76).

Laser Capture Microdissection Studies. Laser capture microdissection can separate malignant breast cells from surrounding lymphocytes before PCR testing. Fina et al. (29) amplified EBV from the malignant epithelial cells of two microdissected tumors. In 115 microdissected tumors, McCall et al. (27) amplified EBA in only two tumors: one subsequently was negative for EBV by ISH for EBER and IHC for LMP1, while the other was positive by ISH for EBER in tumor cells and in 50–75% of normal epithelium, and IHC for LMP1 suggested non-specific staining (27). In tumor cell-rich areas dissected from two
cases, Xue et al. (55) showed EBNA1 and BART expression similar to that observed in whole tissue sections, suggesting that EBV was localized to tumor cells. Murray et al. (75) found no EBV in microdissected tumor cells of 19 tumors PCR-positive for EBV at low levels.

Multiple Analytical Methods. Some groups used multiple assays to evaluate all tumors under study (Table 2). Using seven methods, Chu et al. (31) found 40% of tumors EBV-positive by at least one and only 13% positive by more than one assay; however, in tumors positive by ISH for both EBER and EBNA1, the EBV-infected cell populations did not overlap, suggesting that analytical problems and/or biological diversity in the viral genes and gene products. Deshpande et al. (32) had uniformly negative results for ISH and IHC targeting various viral proteins. Herrmann and Niedobitek (34) found positive results only with PCR.

Epidemiological Considerations. Epidemiological issues have been inconsistently addressed in research into EBV and breast cancer and often ignored in the interpretation of variable results. A few patient series have been population representative, but most have been from clinical institutions, making their representativeness uncertain. Only Fina et al. (29) selected study subjects with consideration of EBV-related cancer patterns. Most other studies have involved US or European groups, which could mean limited geographic, socioeconomic, and racial diversity and, in turn, exclusion of the populations likely to be at high risk of EBV-related disease, according to the epidemiology of other EBV cancers (35). In addition, given the apparently low prevalence of EBV in breast cancer, the sample sizes of many of the 20 studies to date (Table 2) have been too small (median of 48 patients, mean of 80) for informative epidemiological analyses.

Several groups examined the relationship of EBV presence to clinical and demographic characteristics (Table 3). Four studies found a stronger EBV association in tumors with features of more aggressive disease (25, 29, 31, 75). Bonnet et al. (25) noted EBV prevalences of 27%, 44%, and 66% for tumor grades I, II, and III (P = 0.03); 45% and 79% for ER-positive and ER-negative tumors (P = 0.01); and 54%, 32%, and 72% for 0, 1–3, and 4+ positive nodes (P = 0.01); as well as virus in metastases of EBV-positive primary tumors. Fina et al. (29) noted a suggestive association of EBV prevalence with tumor grade (22% versus 36% for grades I and III) and a significantly higher tumor viral load in patients from geographic regions with higher rates of NPC (Algeria, Tunisia) than from regions with lower NPC rates (northern France, Holland, Denmark), suggesting that factors increasing viral load, such as poor socioeconomic conditions (35), could impact EBV presence in breast cancer. Chu et al. (31) found some evidence associating EBV positivity with higher grade tumors (0/2, 3/36, and 3/10 in grades I, II, and III by EBER1 ISH). Grinstein et al. (33) reported IHC evidence of EBNA1 in carcinoma in situ, consistent with EBV presence in precursor lesions. Murray et al. (75) noted positivity for the EBNA1 2B4-1 antibody significantly correlated with histology (75% in medullary, 8% in lobular), tumor size (20% versus 43% for ≤20 mm) and grade (10% versus 47% for grades I and III), number of positive lymph nodes (24% versus 50% for 0 versus >3), ER status (21% and 61% for positive and negative), and disease stage. Chu et al. (31) noted higher EBV positivity in women under than over age 50 (50% versus 35%), but the two studies considering menopausal status found no association with EBV positivity (25, 29).

Interpretation of Findings to Date

Since 1993, EBV has been found in a proportion of breast carcinomas by numerous studies (15, 20, 21, 25–27, 29, 31, 33, 34, 55, 75) and been completely undetected by others (19, 22–24, 28, 30, 77), using a variety of analytical approaches. Virus has been found most consistently and in the highest prevalence (approximately 10–50%) using PCR, and histochemical methods have produced evidence of viral DNA or proteins within malignant breast epithelial cells that suggests a pathogenic role for EBV. While the prevalence of EBV-positive cases from the more definitive morphology-based methods is difficult to determine, estimates range from 1% to 19% from EBER-ISH studies and from 4% to 51% for EBNA1 IHC studies. Some studies considering clinical or demographic characteristics have found that EBV-positive breast cancers may be more aggressive, have higher viral loads, or occur in younger women (25, 29, 31, 75).

The inconsistency and variability of these findings is challenging to reconcile, leading some researchers to conclude that EBV is not involved in breast cancer (23, 27, 30, 31, 34). However, while the accumulated evidence indicates that the EBV-breast cancer association is not conventional by previous standards, the apparent variability should not, itself, rule out a role for EBV in breast tumorigenesis or cancer progression, because it is influenced to some extent by three unresolved issues: (a) the marked variation in EBV prevalence, even among studies using similar methodologies (25, 26, 32); (b) the possibility of false-positive or -negative results due to lymphocyte-derived EBV, amplicon contamination or cross-reactive immunostains, and inadequate assay sensitivity; and (c) absence of the previously assumed biological “hallmarks” of an EBV-cancer association, including uniform expression of EBERs in all cells of virus-associated tumors. Therefore, it is important to consider an interpretation of no association in the context of these limitations, as follows.

1. Variable EBV prevalence. The presence of EBV in only a proportion of cases, and the variability in this proportion across studies, are consistent patterns for all EBV-related cancers except perhaps undifferentiated NPC and African Burkitt lymphoma (35). Among breast cancer studies using the same viral detection assays, such variability may occur due to differential inclusion of patient subgroups that ultimately may prove susceptible to this form of the disease. In a multi-center study of Hodgkin lymphoma, a considerable proportion of inter-study variability in EBV prevalence was eliminated once epidemiological differences among populations were considered (73).
Table 2. Characteristics and results of studies to detect EBV in breast cancers

<table>
<thead>
<tr>
<th>First author, year</th>
<th>Breast histology</th>
<th>Region</th>
<th>N</th>
<th>Tissue preparation</th>
<th>Assay</th>
<th>EBV positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gaffey, 1993 (19)</td>
<td>Infiltrating ductal, medullary carcinoma</td>
<td>US</td>
<td>35</td>
<td>Paraffin</td>
<td>PCR for EBNA1</td>
<td>0%</td>
</tr>
<tr>
<td>Horiuchi, 1994 (20)</td>
<td>Lymphoepithelioma-like carcinoma</td>
<td>Japan</td>
<td>3</td>
<td>Paraffin</td>
<td>PCR for BamHIW ISH for BamHIW</td>
<td>66%</td>
</tr>
<tr>
<td>Luqmani, 1995 (21)</td>
<td>Invasive carcinoma, various</td>
<td>England</td>
<td>28</td>
<td>Paraffin</td>
<td>Nested PCR for BamHIW ISH for EBER PCR for BamHIW IHC for LMP1</td>
<td>54% Focal positive 0%</td>
</tr>
<tr>
<td>Lespagnard, 1995 (22)</td>
<td>Medullary carcinoma</td>
<td>Belgium</td>
<td>10</td>
<td>Paraffin</td>
<td>PCR for BamHIW ISH for EBV DNA IHC for LMP1 PCR for BamHI-W PCR for EBERs ISH for BamHIW DNA</td>
<td>21% 17/34 (50%) of selected cases 6/19 (32%) 0% 0% 0%</td>
</tr>
<tr>
<td>Labrecque, 1995 (15)</td>
<td>Invasive carcinoma, various</td>
<td>England</td>
<td>91</td>
<td>Frozen, paraffin</td>
<td>PCR for BamHIW ISH for EBER1 DNA IHC for LMP1 PCR for EBER1 PCR for EBER2, BZLF1, LMP2 ISH for EBER1</td>
<td>51% 0/3 PCR+ 7/7 (100%) 9/9 (100%) PCR+ 1/5 (20%) PCR+ 0/5 PCR+</td>
</tr>
<tr>
<td>Chu, 1998 (23)</td>
<td>Invasive ductal carcinoma, some with medullary features</td>
<td>Taiwan</td>
<td>60</td>
<td>Paraffin</td>
<td>PCR for BamHIW ISH for EBER1 RNA IHC for EBNA2 IHC for LMP1 ISH for EBER1 ISH for EBER1</td>
<td>0% 0% 0% 0% 0%</td>
</tr>
<tr>
<td>Glaser, 1998 (24)</td>
<td>Invasive carcinoma, various</td>
<td>US</td>
<td>107</td>
<td>Paraffin</td>
<td>PCR for EBER2, BZLF1, LMP2 IHC for EBNA1 Southern blot for BamHIW IHC for EBNA1</td>
<td>51% 0/3 PCR+ 7/7 (100%) 9/9 (100%) PCR+ 1/5 (20%) PCR+ 0/5 PCR+</td>
</tr>
<tr>
<td>Bonnet, 1999 (25)</td>
<td>Invasive ductal, lobular, other carcinomas</td>
<td>France</td>
<td>100</td>
<td>Frozen, paraffin</td>
<td>PCR for BamHIW ISH for EBER1 RNA IHC for EBNA1 PCR for EBER1/2 Microdissection and PCR for EBNA1 ISH for EBER IHC for LMP1</td>
<td>2/115 (2%) 1/2 (50%) PCR+ 1/2 PCR+ (atypical pattern) 0%</td>
</tr>
<tr>
<td>Brink, 2000 (26)</td>
<td>Carcinoma</td>
<td>Holland</td>
<td>24</td>
<td>Frozen</td>
<td>PCR for BamHIW PCR for LMP1 rtPCR for BamHI1A RNA rtPCR for EBNA1 RNA IHC for EBNA1 ISH for EBER1 PCR for EBER1</td>
<td>21% 2/5 (40%) PCR+ 0/5 PCR+ 0/5 PCR+ 1/5 (20%) PCR+ 0/5 PCR+</td>
</tr>
<tr>
<td>McCall, 2001 (27)</td>
<td>Intraductal, invasive, or metastatic carcinoma</td>
<td>US</td>
<td>115</td>
<td>Paraffin</td>
<td>Microdissection and PCR for EBNA1 ISH for EBER IHC for LMP1</td>
<td>2/115 (2%) 1/2 (50%) PCR+ 1/2 PCR+ (atypical pattern) 0%</td>
</tr>
<tr>
<td>Dadmanesh, 2001 (28)</td>
<td>Lymphoepithelioma-like carcinoma</td>
<td>Italy</td>
<td>4</td>
<td>Paraffin</td>
<td>ISH for EBER1 ISH for EBER1</td>
<td>0% 0%</td>
</tr>
<tr>
<td>Kijima, 2001 (30)</td>
<td>Carcinoma</td>
<td>Japan</td>
<td>61</td>
<td>Paraffin</td>
<td>ISH for EBER1 PCR for EBER1 ISH for EBER1</td>
<td>0% 32% 10/20 (50%) PCR+ 2/2 (100%)</td>
</tr>
<tr>
<td>Fina, 2001 (29)</td>
<td>Invasive ductal carcinoma</td>
<td>North Africa, Europe</td>
<td>509</td>
<td>Frozen, paraffin</td>
<td>PCR for EBER1 ISH for EBER1 PCR for EBER1 PCR for EBNA1 ISH for EBNA1 PCR for LMP1 ISH for BZLF1 PCR for EBER1 PCR for EBNA4 (EBNA-3b) PCR for LMP1 Southern blot for EBV clonality</td>
<td>25% 0% 0% 10% 10% 10% 10% 0/6 PCR+</td>
</tr>
<tr>
<td>Chu, 2001 (31)</td>
<td>Invasive carcinoma</td>
<td>US</td>
<td>48</td>
<td>Frozen, paraffin</td>
<td>IHC for EBNA1 IHC for LMP1 IHC for BZLF1 ISH for EBNA1 PCR for EBER1 PCR for EBNA1 PCR for LMP1 Southern blot for EBV clonality</td>
<td>25% 0% 0% 10% 10% 10% 10% 0/6 PCR+</td>
</tr>
</tbody>
</table>

(Continued on the following page)
2. False-positive and -negative analytical test results. For breast cancer studies, PCR may overstate EBV association because it cannot indicate what types of cells the virus has infected. McCall et al. (27) showed that EBV positivity by PCR was correlated with the number of PCR cycles and consistent with the level of EBV expected in normal latently infected lymphocytes. Yet, several positive studies found no EBV in lymphoid infiltrates of breast cancers, even near EBV-positive tumor cells (15, 29), or in healthy breast tissue adjacent to EBV-positive tumors (15, 21, 25, 29). PCR results have been confirmed with Southern blot hybridization, the sensitivity of which is considered too low to detect virus in infiltrating lymphocytes (25), and in some PCR-positive tumors, EBV presence was confirmed in malignant cells isolated by microdissection (27, 29, 55). PCR raises concerns about false-positive results due to amplicon contamination (44), but Bonnet et al. (25) had positive findings in independent amplifications of three different EBV genes. For immunohistochemical studies, false-positive results may occur due to antibody cross-reactivity with unintended antigens or to high background staining in the detection system. Cross-reactivity is a particular concern for IHC targeting EBNA1 and LMP1 (26, 31, 44), and other immunohistochemical assays (e.g., BZLF1) are even less well studied with regard to their analytical performance characteristics.

On the other hand, EBV may be missed even when present because of test insensitivity or absence in the infected tissue of the particular viral factor being targeted. For PCR, the apparent variation in EBV prevalence with the target gene and the PCR conditions illustrates the impact of analytical strategy on the extent of the viral association identified. In addition, negative results are not reliable unless a control assay has been used to insure that amplifiable DNA (or cDNA) is extracted. Hybridization to EBV DNA may be hampered by partial deletion or polymorphisms of viral DNA. Studies targeting RNA or protein may be falsely negative because of inadequate antigen retrieval procedures, inadequate signal-to-noise ratio, or problems with tissue fixation or preparation (78).

3. Absence of EBV-cancer association hallmarks. EBERs have been a consistent feature of EBV-related cancers (Table 1) but are rarely found in breast tumor cells (37). However, EBERs are not found in all NPCs (79).
or in some Burkitt and Hodgkin lymphomas that are LMP1-positive or positive by PCR (44, 73). Breast tumors may express EBERs less abundantly than other malignancies (15, 25) or sustain a previously unrecognized form of EBV infection characterized by EBER down-regulation (31, 80), as recently suggested for liver adenocarcinomas (81, 82). EBER coding sequences also may be deleted from the EBV genome. The apparent absence of EBV from many malignant cells of infected tumors suggests that the virus was not present in the progenitor clone. However, EBV is not detected in all tumor cells of certain EBV-associated B- and T-cell lymphomas (83, 84). EBV-associated tumors may be only focally infected (44, 79, 83, 84), or the virus may be missing from some cells because of a “hit-and-run” mechanism (15), in which EBV played a critical role in carcinogenesis but subsequently was partially or entirely lost (85–87), or because of other mechanisms of epithelial cell immortalization (54). EBV might not be detected in some cells because of alterations associated with its integration into host chromosomal DNA (88), or because the viral targets are present at levels that span the sensitivity threshold of the assay (25, 34); McCall et al. (27) noted that most PCR-positive results targeted the BamH1W internal repeats, which are about 10 times more prevalent than single-copy viral genes. EBV infection also may occur in selected tumor cells after malignant transformation (15, 31) or impact tumor pathogenesis by driving already established malignant behavior (15, 31). This possibility is supported by the observation that, in vitro, EBV is better able to enter actively dividing breast epithelial cells (70); by the reported association of EBV with breast tumor aggressiveness and metastases (25, 29, 31, 75); and by the possibility of microcompetition, in which EBV DNA, once in a cell, could act alone, even if no viral genes were expressed, by binding and sequestering cellular transcription factors available to influence cellular gene expression (89).

### Future Direction

Given the technical problems in detecting EBV in breast cancer, the possibility of yet undescribed forms of EBV pathogenesis in epithelial tumors, and the limited epidemiological consideration to date, definitive judgment regarding EBV relatedness of this cancer cannot yet be rendered. Recent technologic advances now make it feasible to better tackle the methodological challenges of detecting EBV in breast cancers. Consequently, a critical next step in understanding this relationship is to apply detection strategies that are sensitive and specific for EBV and able to localize the EBV to particular benign or malignant cells within the tissue. A recent National Cancer Institute recommendation specifies an approach combining real-time quantitative PCR, which allows measurement of the amount of viral DNA in archival tissue samples, with laser capture microdissection to improve localization of viral DNA to benign or malignant components of a tissue sample (90). Ideally, the real-time PCR measurement of viral load should be reported as a ratio to an endogenous gene so as to adjust for the

### Table 3. Relationship of EBV-positive breast cancer to tumor and patient characteristics,* by study

<table>
<thead>
<tr>
<th>First author, year</th>
<th>Region</th>
<th>N</th>
<th>Tumor characteristics</th>
<th>Patient characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Histology</td>
<td>Grade</td>
</tr>
<tr>
<td>Gaffey, 1993 (19)</td>
<td>US</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horiuchi, 1994 (20)</td>
<td>Japan</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luqmani, 1995 (21)</td>
<td>England</td>
<td>28</td>
<td>ductal</td>
<td></td>
</tr>
<tr>
<td>Lespagnard, 1995 (22)</td>
<td>Belgium</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Labrecque, 1995 (15)</td>
<td>England</td>
<td>91</td>
<td>x ¹</td>
<td></td>
</tr>
<tr>
<td>Chu, 1998 (23)</td>
<td>Taiwan</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glaser, 1998 (24)</td>
<td>US</td>
<td>107</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Bonnet, 1999 (25)</td>
<td>France</td>
<td>100</td>
<td>x</td>
<td>higher</td>
</tr>
<tr>
<td>Brink, 2000 (26)</td>
<td>Holland</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>McCall, 2001 (27)</td>
<td>US</td>
<td>115</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dadmanesh, 2001 (28)</td>
<td>Italy</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kijima, 2001 (30)</td>
<td>Japan</td>
<td>61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fina, 2001 (29)</td>
<td>Europe</td>
<td>509</td>
<td>(higher)</td>
<td>x</td>
</tr>
<tr>
<td>Chu, 2001 (31)</td>
<td>US</td>
<td>48</td>
<td>x</td>
<td>(higher)</td>
</tr>
<tr>
<td>Grinstein, 2002 (33)</td>
<td>US</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deshpande, 2002 (32)</td>
<td>US</td>
<td>43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herrmann, 2003 (34)</td>
<td>Germany</td>
<td>59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xue, 2003 (55)</td>
<td>UK</td>
<td>15</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Murray, 2003 (75)</td>
<td>UK</td>
<td>153</td>
<td>medullary</td>
<td>higher</td>
</tr>
<tr>
<td>Preciado, 2004 (74)</td>
<td>Americas</td>
<td>102</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*EBV association described by term: e.g., “higher” means EBV-positive tumors were of higher grade.

¹Menopausal status.

²‘‘x’’ means study results were stratified by this factor.

³Three-year survival (no association).

⁴( ) association not statistically significant.

Higher viral load in regions with higher rates of nasopharyngeal carcinoma.

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extraction efficiency and DNA quality in the sample. Irrespective of the assays selected, laboratory error must be avoided by validating assay sensitivity and specificity before interpreting results on EBV relatedness; by investigating assay cross-reactivity with uninfected tissue and with tissue infected by other pathogens; and by using “no template” controls to check for amplicon contamination in PCR studies, “no antibody” controls to check for non-specific immunohistochemical stains, and uninfected tissues or tissues infected by other pathogens to test assay specificity for EBV. Furthermore, appropriate laboratory strategies must be applied to breast cancer cases that are collected and analyzed to test for EBV infection. These strategies are based on prior studies of EBV in breast and other cancers.

At present, public health and clinical considerations justify such research. A link of EBV with breast cancer etiology or progression, even in a minority of patients, would mean a significant population attributable fraction, given the high incidence of breast cancer. If EBV were linked with breast cancer in only 10% of patients, as suggested by recent localizations studies, this group would comprise an estimated 33,000 newly diagnosed women in the US each year (91). Convincing positive results of analytically well-conducted studies would support further work to characterize the epidemiology of EBV-related breast cancer and identify the high-risk patient subsets, to examine the possible interaction of EBV presence with biomarkers like hormone receptor status and markers of immune function, and to understand the role of EBV in development or maintenance of cancer cells. In the clinical realm, identification of an association of EBV with breast cancer could have implications for new treatments, such as the use of EBV as an antigenic target for stimulated cytotoxic T cells (16), and potential for improved clinical management if EBV serum load could be used to evaluate treatment efficacy as is done for NPC and post-transplant lymphoproliferations (92, 93). If patients with EBV-related breast cancers prove to have more advanced disease, viral detection could be relevant to planning the course of treatment. With evidence for an association with EBV, breast cancer detection and prevention also could be enhanced: early detection of EBV-related breast cancers might be feasible based on EBV-specific test strategies, and prevention efforts could draw from ongoing clinical trials for vaccination against EBV (18, 80). Finally, negative results from reliable detection systems could lead to a more readily accepted conclusion of no direct relationship between this common virus and common cancer.

References

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