Epstein-Barr Virus Detection in Ductal Carcinoma of the Breast

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Epstein-Barr virus (EBV) has been strongly linked with African Burkitt’s lymphoma and nasopharyngeal carcinoma (NPC) and has recently been associated with breast cancer (1). Nine studies of EBV in breast cancer with the use of different methods (1–9) present conflicting results.

In the current study, microdissected ductal breast carcinomas were tested for EBV. Only one of 115 cases was positive. This finding stands in contrast to results of studies of ductal carcinoma showing up to 48% EBV positivity by polymerase chain reaction (PCR).

Bonnet et al. (1) found 51 of 100 breast cancers of all types to be PCR positive for EBV. EBV positivity was also shown by Southern blot analysis and immunostain in a subset of cases, but in situ hybridization (ISH) for EBER was negative. A similar pattern has been observed previously (2).

Our study included 115 breast cancers, chosen for having microdissectable normal, as well as intraductal, invasive, or metastatic tumor components. Intraductal carcinoma specimens were obtained from 84 patients, invasive tumor in 106 patients, metastases in 50 patients, and recurrences in six. Normal lymph node or other normal tissue was also collected, for a total of 361 samples (10). Institutional approval was received for the conduct of the study.

Samples were analyzed by 5′-nuclease PCR for the EBNA1 gene. Primers were CTGGGACGARGCTCCG-TAMRA (nucleotides 662–687). PCR was performed in 50 μL with 200-nm primers and a 100-nm probe. Cycling conditions were as follows: 50 °C for 2 minutes and 95 °C for 10 minutes, followed by 50 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. The assay detected a single viral copy when titrated against dilutions of a quantified EBV DNA control (Advanced Biotechnologies, Columbia, MD). DNA amplifiability was assessed with the use of the HER2 gene as a control. Primers were ATGCAGTTGCAAGGTATGC (nucleotides 977–997; GenBank M12036) and GGAAGCCCATGTAGAC-CTTCT (nucleotides 1098–1120), and the probe was VIC-CCGGAGCAAC-CCTATGCTCCA-TAMRA (nucleotides 1021–1045). A kit was used for EBER ISH (Biogenex, San Ramon CA). Immunostaining for LMP1 was performed with monoclonal antibody clones CS1–4 (DAKO A/S, Glostrup, Denmark).

Amplifiable DNA was obtained from 278 (77%) of 361 samples. Of these, only six samples were positive for EBNA1. EBV-positive blocks were analyzed by EBER ISH and LMP1 immunohistochemistry. Three were lymph nodes—two from cases without metastases and one from a case with metastatic carcinoma. All of these contained occasional EBER- and LMP1-positive lymphocytes; the metastatic tumor itself was negative for both EBER and LMP1.

The other three positive specimens were from one patient. In this case, both EBER and LMP1 staining demonstrated that EBV was present in 50%–75% of normal ductal epithelium, as well as in the intraductal and invasive carcinoma components. The expected nuclear location of EBERs was observed (11,12). LMP1 staining was predominantly nuclear, in contrast to positive controls showing typical membrane staining (13,14).

The predominant human EBV is the B95.8 strain. Variant strains may be responsible for the epithelial tropism of NPC (15). Theoretically, variants might escape PCR detection, but a study of NPC patients with the use of primers in the variable region of EBNA1 still detected EBV in all 50 cases (16). The sensitivity of our assay was confirmed in an ethnically diverse series of NPC patients. For 13 cases with amplifiable DNA, nine (69%) were positive for EBV. Therefore, it is unlikely that our negative results were due to undetectable EBV variants.

Most investigations have used fixed tissue; however, two studies (1,3) used fresh frozen tissue and detected EBV by PCR plus either ISH or immunostain in 25%–48% of ductal carcinomas. Luqmani et al. (2) performed PCR on 48 frozen and 12 fixed samples and found them to be positive in 39% and 33%, respectively. This modest gain in sensitivity in fresh tissue is insufficient to explain the discrepancies in the literature. The PCR target is another potential source of variation. Most studies yielding positive results used targets in the long internal repeat (IR1), where PCR is 10 times more sensitive than for the EBER gene (1).

While incomplete reporting and differences in methodology or diagnoses prevent strict meta-analysis, aggregate statistics for EBV detection were compiled. For all studies, PCR detected EBV in 23.8% of all patients and histologies. Given the sensitivity of PCR and the persistence of latent lymphocytic infection, this result is unsurprising. Stable, latently infected lymphocytes contain multiple EBV copies (17), and the overall positivity rate in breast cancer is no higher than that often observed in normal tissues (18–23).

Microdissection is a unique advantage of this study, thereby reducing benign lymphocyte contamination. The purity of these microdissections from stromal contamination has been demonstrated by loss-of-heterozygosity analyses (10). The other published investigations (1–9) used lysates from whole tumors. One such study (5) demonstrated by ISH that EBV PCR positivity in breast cancer was due to latently infected lymphocytes.
Because PCR cannot distinguish between neoplastic cells and latently infected lymphocytes, high rates of positivity in breast carcinomas indicate a lack of specificity. LMP1 immunostain and EBER ISH, which provide greater specificity (24), were positive in only 6%–7% of breast cancer cases reported in the literature. In contrast to conventional PCR, the use of the 5'-nuclease PCR system should improve specificity by its use of a sequence-specific probe in the reporting system.

The chief determinant of the EBV detection rate by PCR in breast cancer may be the number of cycles used. The highest rates (51% and 45%) were in large studies employing 80 cycles of nested PCR (1,2). Linear regression was performed, weighted by the number of cases in each study. After the first 28 cycles, each additional cycle was associated with an increased EBV detection of 0.85%. With a correlation coefficient of $r = .780$, this model is highly significant (two-sided $P < .001$) and accounts for 61% of the variance (Fig. 1).

Proportions, like EBV detection percentages, often have greatest variance in the midrange. Hence, an angular transformation was also performed (25). The proportion of positive cases in each study was converted to its square root, and then the arcsine of the square root was calculated. Weighted linear regression still shows a highly significant relationship (two-sided $P < .001$) between EBV detection and the number of PCR cycles. With the use of this angular transformation, the $y$ intercept was 14 cycles, after which EBV detection increased 0.34% per cycle. The correlation coefficient was $r = .733$, accounting for 54% of the variance.

At this time, a preponderance of evidence suggests that the high PCR positivity in previous studies is largely an artifact of latently infected lymphocytes. Furthermore, it seems unlikely that very low viral copy numbers detected in nested PCR would be pathologically significant. In our study, only one among 115 breast cancers had EBV present by EBNA1 PCR and in malignant cells by EBER ISH and LMP1 immunostain. The pathophysiologic significance of this finding is unclear because the possibility remains that EBV occasionally persists without any role in pathogenesis. Although it is possible that EBV causes cellular transformation in some cases, current evidence argues against an important role for this virus in breast cancer pathogenesis.

REFERENCES


NOTES

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