Epstein-Barr Virus in Epithelial Cell Tumors: A Breast Cancer Study

Louise G. Labrecque, Diana M. Barnes, Ian S. Fentiman, and Beverly E. Griffin

Department of Virology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London, United Kingdom. ... Imperial Cancer Research Fund Clinical Oncology Unit, Guy's Hospital, London, United Kingdom SE1 9RT ¡D. M. B., I. S. F., D. M. B., I. S. F.

Abstract

The human herpes virus Epstein-Barr (EBV) is clearly associated with African Burkitt's lymphoma and the undifferentiated form of nasopharyngeal carcinoma, although its role in oncogenesis is still poorly defined. Recently EBV has been implicated in other types of lymphomas, as well as in some nonlymphomatous neoplastic processes. Its possible association with human breast cancer has been investigated here. DNA from 91 cases of breast carcinoma and blood samples from the same patients were amplified with the PCR over a region in the EBV BamHIW major repeat sequence following a single-step amplification protocol. Nineteen samples (21%) were found to be positive; 10 samples of blood (only 3 of them from patients with EBV-positive tumors) were found by the adopted protocol to contain EBV DNA. Another series of PCR amplifications using primers covering a unique (nonreiterated) fragment in BamHI encoding the EBERs (two short nonpolyadenylated RNAs generally highly expressed in cells latently infected with EBV) confirmed these findings. A good correlation between the two sets of experiments was observed, and only five differences in results were obtained on samples tested. In situ hybridization was carried out using BamHIW biotinylated DNA probes or EBER-1 digoxigenin-labeled riboprobes with the aim of confirming as well as localizing the signal to the epithelial cell. Twelve sections (63%) among the PCR-positive samples were found positive by in situ hybridization with the DNA probe, and six (31.5%) sections were found with the RNA probe. Twenty-one samples from benign breast tumors or normal breast tissue were used as controls for PCR amplification in this study, none of which was found positive. This is the first known report showing positive results for EBV in breast cancer. No statistical association was found in these studies between the presence of EBV and the histological type of the tumor, however. Its role therefore remains for the moment unknown, as well as does the significance of the association of EBV with only a subset of the cases.

Introduction

EBV is an oncogenic DNA virus associated with African Burkitt's lymphoma (1), some AIDS-associated lymphomas (2), and non-Hodgkin's lymphomas seen in graft patients (3). It is also linked with a subpopulation of Hodgkin's lymphomas (4), as well as with the undifferentiated (5) and some differentiated (6) types of nasopharyngeal carcinomas. The cancers with the strongest viral association, African BL and NPC, occur in very different populations. BL is found mainly in young children in sub-Saharan Africa, and NPC is particularly prevalent in middle-aged Chinese men, but both tumors, with high frequency, carry EB viral genetic material and exhibit a restricted pattern of expression of the viral genome (7). EBV has been linked occasionally with other types of malignancies, including a pseudomyelomatous lung carcinoma (8) and gastric adenocarcinoma (9–10). No etiological association between the presence of EBV and disease has been proved thus far, however, and its exact role in transformation is unknown. Nor is it clear whether persistence of the viral genome is mandatory for maintenance of the cellular phenotypic alteration.

EBV is ubiquitous in the population; more than 90–95% of the adult world population shows serological evidence of past infection (11). Receptors for the virus have been identified in epithelial cells from the oropharynx and in cells lining the uterus (12–13), and it is postulated that the virus can directly infect such epithelial cells; in vitro, a subfragment of the EBV genome can immortalize epithelial cells (14–15). Primary non-Hodgkin's lymphomas may occur in the human breast (16), and in the African (endemic) form of BL the breast can also be a target site (17–18). In Africa also a high incidence of breast cancer has been reported in men in the sub-Saharan area called the "breast cancer belt" (19). This zone is included in the malaria belt where the highest incidence of EBV-associated BL is found. In Brazil, where there are relatively more EBV-associated lymphomas than in Western countries, there is (in Recife, Brazil) the highest observed incidence of breast cancer in males in the world. Stimulated by these data and the endemicity of breast cancer in Western societies (20), we screened a large bank of breast cancer DNAs, in parallel with blood samples obtained from the same patients, to look for evidence of a link between EBV and mammary carcinoma. Were such an association to be found, particularly in a subset of the malignancies, it might help to explain the diversity and the sometimes unexpected behavior of the disease, that are not compatible with markers such as hormone receptor status or presence of metastases at presentation.

This paper demonstrates that EB viral genetic information exists in at least 20% of the cases of breast tumor assessed. The methods we used were PCR amplification, using two different pairs of primers covering the BamHIW repeat region of the viral genome (21) and a subregion of BamHIC, respectively (Fig. 1), that encode the EBERs (22), two short, nonpolyadenylated RNAs highly expressed in cells latently infected with EBV (23), and in situ hybridization (with similar probes) to confirm by another route the results obtained by PCR, as well as to localize the viral signal in the tumor sections.

Materials and Methods

DNA Samples. The study was conducted on paired samples of DNA from blood and primary breast carcinomas taken from patients attending the Imperial Cancer Research Fund Clinical Oncology Unit at Guy's Hospital between 1987 and 1989. The DNA was prepared using an automated DNA extractor and had been stored in the DNA bank of the unit. There was no evidence of sample cross contamination as assessed by PCR studies looking for several different oncogenes. Tumors were typed according to the WHO classification (24) and were graded according to the criteria of Bloom and Richardson as modified by Ellis and Elston (25). Twenty-one samples of normal (1 case) or benign breast tumors with no atypia (20 cases) were used as controls. The DNA was extracted according to a standard protocol (26), and the absorbance was measured at 260 and 280 nm using a UV spectrophotometer.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: EBV, Epstein-Barr virus; BL, Burkitt's lymphoma; NPC, nasopharyngeal carcinoma; ISH, in situ hybridization; EBER, E15-encoded small RNA.

4 N. Lemoine, personal communication.
EBV IN EPITHELIAL CELL TUMORS

EBV genome co-ordinates (kb)

| 0 | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 | 140 | 150 | 160 | 170 |

BamHI restriction map of EBV genome

BamHI Rsi repeats

Fig. 1. BamHI restriction map showing the 2 fragments chosen for PCR amplification (shaded). kb, kilobase.

BamHIW PCR. The primers designed, called LLW1 and LLW2, cover the IR1 repetitive region of the viral genome and are localized between base pairs 505 and 740 in the BamHIW EBV fragment. [LLW1: 5'-CCATCGT AACCTCGGCTCGAG-3', a restriction site (underlined) introduced by modification in position 11 (nucleotide 515); and LLW2: 5'-GGCTTTG-3', a restriction site (underlined) already present. The entire amplified fragment would be 236 base pairs.

BamHIC PCR. The primers designed, called EBER A and B, cover both EBERs (1 and 2) and the region amplified, are localized between nucleotides 6623 and 7123 in the fragment, and cover 501 base pairs. EBER A: 5'-AACCTCAGACCTACGCT-3'; and EBER B: 5'-TAGCGGACAAGCCGAAATC-3'.

PCR Protocol. Amplification was carried out according to a standard protocol (27). The PCR mix (100 μl) was composed of 21.5 μl water, 10 μl 10X reaction buffer (100 mM Tris-HCl (pH 8.3)-500 mM KCl-15 mM MgCl2-0.1% gelatin), 16 μl of 1.25 mM each deoxynucleotide triphosphate [made up with individual 10 mM stock solutions of each type (dATP, dCTP, dGTP, and dTTP) in double distilled sterile water (pH 7) adjusted with 3 M NaOH and the concentration measured spectrophotometrically (final concentration 200 μM of each deoxynucleotide triphosphate)], 1 μl of each primer (final concentration 1 μM), and 0.5 μl Taq DNA polymerase (5 Units/μl; 2.5 units/tube). Fifty μl of the mix was added to each PCR tube and covered with 3 drops of sterile mineral oil. One μg of the template DNA was then added to a total volume of 50 μl of water. The PCR reaction was carried out using a denaturation cycle at a temperature of 95°C for BamHIW (94°C for BamHIC) for 40 s, annealing temperature of 59°C for BamHIW (56°C for BamHIC) for 1 min, and an extension cycle at 72°C for 1.5 min. Thirty-35 cycles were performed, the last one being extended to 5 min. The preparation of the mix, the PCR reaction, as well as the manipulation of the PCR products obtained were carried out in different rooms to avoid any risk of cross-contamination by carrying over of materials from other experiments. The products obtained were separated by electrophoresis on an agarose gel and then transferred by Southern blotting to a nylon membrane [Biotune B using a NeoH-denaturation technique following the instructions of the manufacturer (Pal)] and crossed-linked by UV or heat treatment. Hybridization was carried out either with a [32P]dCTP-radio-labeled DNA probe (made from the BamHIW fragment of the EBV genome in Bluescript vector, Stratagene) or with a purified EBER-PCR product (obtained by PCR amplification of 1 μg of Namalwa DNA, then purified by electrophoresis, phenol extraction, and ethanol-sodium acetate precipitation). The radio-labeling, using 40 μC of [32P]dCTP and 25 ng of DNA was carried out with the Multiprint kit (Amersham, United Kingdom) following the instructions of the manufacturer. In each case, hybridization was carried out at either 68°C overnight in a shaking water bath or using a hybridizer (HB-1, Technne). Membranes were then washed stringently and sequentially in 2X SSC (1X SSC = 0.15 M NaCl, 15 mM trisodium citrate)-0.1% SDS, 1X SSC-0.1% SDS, and 0.2X SSC-0.1% SDS at 68°C. They were then left in contact with X-ray film (FUJI Japan) in an autoradiograph cassette for a variable length of time (1 day-1 week or more) at -70°C before developing.

ISH. The protocol used for DNA ISH is that published (28). Briefly, fresh frozen sections were allowed to thaw to -70°C, treated with HCl (0.02 M), and then with Triton X-100, permeabilized with Pronase (0.25 mg/ml) in Tris-HCl and EDTA at room temperature for 6 min, and digested with RNase A (100 μg/ml) at 37°C for 1 h. Intrinsic biont activity (29) was blocked with avidin followed by an incubation in biotin. The slides were then dehydrated in ethanol after washing in PBS.

Formalin-fixed paraffin-embedded sections were dewaxed in xylene for 10 min (twice), and then soaked in ethanol. They were digested with 4 mg/ml pepsin-HCl (0.1 m) for 60 min at 37°C, following digestion with RNase A, then manipulated as for fresh frozen sections.

The hybridization mix was added to the sections (10-20 μl according to the size) and they were put in a cytospin path box. No coverslip was added. Slides were denaturated in a pressure cooker at 108-121°C for 2-6 min, and incubations performed overnight in a covered glass container placed in a 37°C water bath. Slides were then washed in 2X SSC (three times) and in 0.2X SSC at 42°C. The cytochemical visualization was made with streptavidine alkaline phosphatase (direct technique) or with mouse streptavidine, biotinylated anti-mouse, and streptavidine alkaline phosphatase (indirect technique) using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as dye solution.

The protocol for RNA ISH was a modification of that used for DNA. An EBER1 recombinant fragment, in a Blue Scribe vector harboring T3 and T7 promoters (kindly provided by Dr. Richard Ambinder, Johns Hopkins Medical School, Baltimore, Maryland) (23) was used to generate sense and antisense probes. A shorter digestion time of the fixed sections (30 min) with pepsin-HCl than that time previously used for DNA ISH was performed. No RNase A treatment was carried out and the material was acetylated prior to hybridization. No DNA was used in the probe mix which was prepared with deionized water treated with diethyl pyrocarbonate. The sections and hybridization mix were heated at a lower temperature than for the DNA ISH (95-108°C), and hybridization was carried out overnight in a 56°C water bath. Following hybridization, slides were washed in 2X SSC-0.1% SDS-0.2X SSC-0.1% SDS at 42°C (30 min), digested with RNase A (100 μg/ml at 37°C for 30 min), then soaked sequentially in buffer 1 (0.1 M maleic acid-0.15 mM NaCl), and then in buffer 2 (buffer 1 in 10% blocking solution (Boehringer-Mannheim) (30 min). Slides were finally incubated in anti-digoxigenin antibody (1 of 300) in 10% normal sheep serum in buffer 2 (60-90 min) at room temperature. An indirect technique could also be used for increased sensitivity, using a mouse anti-digoxigenin antibody (Vector), an anti-mouse biotinylated antibody, and avidine-alkaline phosphatase, but this was accompanied by higher background signals. The visualization step was carried out as in the case of the DNA probe after washing in buffer 1 and equilibration in buffer 3 [0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM MgCl2, as for DNA ISH]. The reaction was allowed to develop overnight.

The two probe systems were extensively tested with different well-characterized EBV-positive and -negative B cell lines before use on breast material.

Results

The results obtained on the presence of the EBV genome in the breast tumor samples are given in Tables 1 and 2: Nineteen (21%) of the 91 samples studied were found to be positive when amplified and probed for the presence of the BamHIW repetitive sequence, with several of them approaching data obtained with the BL-derived cell line, Namalwa (1-2 copies of the EBV genome/cell). No statistical difference, however, was found in the histopathological characteristics (Table 2) between EBV-positive and -negative samples.

Fig. 2 shows a typical gel obtained with materials subjected to PCR after amplification of BamHIW DNA. In all our experiments, a single series of PCR amplifications was performed. This was preferred to the "nested" PCR technique for two reasons: (a) to avoid any risk of contamination by opening a tube containing amplified material; and (b) to avoid too high levels of sensitivity, thereby possibly amplifying spurious material, an obvious risk when dealing with a latent virus. In the experiment presented here, the positive Namalwa control, as well as two of the samples of DNA from breast cancer (numbers 1 and 6), show a band visible by ethidium-bromide staining. The negative control (Molt-4 cells; number 9), as well as the water control (number 11) is negative. Fig. 3 shows a photograph obtained following hybridization of the Southern blot of the gel in Fig 2. The breast samples positive on the gel (numbers 1 and 6) give a highly positive signal, and another sample (number 8) not visually observed on the gel in Fig. 2, was also positive; it did appear, however, after longer exposure. The negative controls remained negative on hybridization (numbers 9 and 11) and the positive control (number 10) scored strongly positive (Fuji film; -70°C.)
patient, and knowing the low level of EBV positive cells among our patients, 3–30 μg of DNA would be required to get a positive signal from infiltrating B lymphocytes in the tumor using our PCR protocol. The fact that positive results were obtained (in the tumors as well as in some cases of peripheral blood) using 1 μg of DNA points to a much higher than expected level of EBV DNA being present in the samples investigated. This could suggest an association between the presence of the virus and the malignant process itself, or vice versa, one being the triggering factor for the other.

As a control for the experiments described above, 21 samples of normal or benign breast tumors were examined. DNAs (1 μg) from these tissues were amplified by PCR (35 cycles) and gels containing the products, with no visibly detectable bands (except in the case of the Namalwa control), transferred by the Southern Blot procedure, and hybridized with the BamHIW probe. None of the samples was found positive even after long exposure to film (data not given), a statistically significant difference with \( P = 0.02 \). When 59 cycles were carried out, 3 samples were found to be weakly positive by long-term autoradiography, suggesting a requirement to avoid extensive amplification by PCR in the case of EBV (or other latent viruses).

To assess whether nonreiterated EB viral DNA could be detected in the tumors, a unique region of EBV DNA, in BamHI C (Fig. 1) was selected for PCR amplification. For this study, 18 samples from the 19 specimens positive by PCR with BamHIW were available for analysis. The data obtained, summarized in Table 1, show that even single-copy regions of EB DNA were detectable in many of the samples.

The incidence of positivity detectable in normal blood samples of EBV seropositive individuals varies greatly among the different studies published in the literature, a very sensitive technique of PCR showing 16% positive (30). The PCR technique used in our studies is of lower sensitivity. To determine the levels of EBV detectable in our procedure, dilutions of DNA from Namalwa cells (averaging 1–2 copies of EBV/cell) were made, amplified in the usual manner, and assessed. The data by gel electrophoresis analysis showed that a minimum of 10 ng DNA was required for visible analysis; on a Southern blot (using the BamHIW probe), 1 ng, corresponding to roughly 200 cells or about 1400–2400 copies of the reiterated sequence, could be detected. For the BL-derived cell line, Raji, with 50 copies of the viral genome, 6 cells should produce approximately the same number of copies of BamHIW. Making the assumption that 50 copies of EBV genome (as in Raji) is an average of what might be expected to be found in infected B lymphocytes of a seropositive
ISH was carried out to assess whether the results obtained by PCR could be confirmed and to localize the signal. All slides were read blind. Fig. 4 shows an ISH of a formalin-fixed paraffin embedded breast tumor, probed with the BamHIW biotinylated DNA probe. Fig. 4a contains the sample hybridized only with biotinylated vector, and Fig. 4b shows a section of the same tumor hybridized with the recombinant BamHIW probe. Although there is minor background noise in Fig. 4a under the experimental conditions used, the level of labeling, localized to the tumor cells in Fig. 4b is much more pronounced, consistent with a much greater hybridization with a probe that carries viral DNA. In Fig. 4b some cells can be observed to have more than one signal, whereas others are apparently negative. (Similar results are obtained when nasopharyngeal carcinomas are examined by this technique.) In all, 12 (63%) sections of the 19 breast samples found to be positive for BamHIW DNA by PCR were confirmed as being positive by ISH; in 4 (21%) cases the signal was weak. The hybridization pattern observed is consistent with the cytology noted in many cases of breast cancer where cell nuclei are asymmetrically distributed within the cell (31). In many cases, technical difficulties associated with ISH were encountered (due to a high level of background “noise,” even after blocking of the intrinsic biotin activity) or the poor retention of the material on the slides. The difference between the sections hybridized with the recombinant viral probe and those hybridized with the vector only (as illustrated) was sufficient to allow a conclusion to be reached in the majority of cases, however. Most of the time the signal was restricted to a small proportion of the cells; the poor retention of the cells on the slides frequently made an accurate evaluation of the proportion of positive cells very difficult (if not impossible) to estimate. Fig. 4c shows an area of a tumor harboring positive cells with a typical particulate pattern. Six of the samples, negative by PCR, were examined by ISH and were found to be negative by this technique also. Twelve other samples, normal (3 samples) and benign (9 samples), breast tumor sections were investigated by ISH and were all found to be negative. There was a strong background in many of these sections but, as such, the levels were similar whether probed with the sense or the antisense probe. Furthermore, background was limited to the intra- and interlobular connective tissue without any epithelial component (not shown).

Fig. 5 shows an RNA ISH on a positive breast cancer with the EBER-1 digoxigenine-labeled riboprobe. Fig. 5a shows an H & E section of the tumor. Fig. 5b contains a section of this same tumor hybridized with the sense riboprobe and Fig. 5c hybridized with the antisense riboprobe. The cells in Fig. 5b show no noticeable signal; the strong signal obtained in Fig. 5c in the positive cells is diffuse and not particulate as in the case of the DNA probe hybridization (an observation also made with different positive EBV B cell lines when hybridized with the same riboprobe in control experiments). Six sections (32%) were positive; other slides were difficult to interpret because of the poor adhesion of the sections to the slides after an overnight incubation at 56°C. Throughout these studies with both types of ISH, we have noted that clusters of cells gave a positive signal, whereas other areas of the same tumors were negative.

**Discussion**

Breast cancer is one of the most prevalent tumors in our Western societies. Many different risk factors have been implicated (32), including an early age at menarche, a late age both at a first complete pregnancy and at menopause, the presence of atypical hyperplasia and a positive family history, lifestyle (such as a diet rich in saturated fat or an intake over 3 units of alcohol a day) and exposure to ionizing radiation. Breast cancer is a “multistep” disease, and a virus could play a role in one of these steps in the pathogenic process. DNA viruses, recognized as oncogenic in humans, include hepatitis B virus (in hepatocarcinoma) (33), papillomavirus (in cervix carcinoma) (33), and EBV (in lymphomas and NPC) (1–7). The association of EBV with African Burkitt’s lymphoma, which presents typically as a tumor mass in the lower jaw in young children from Central Africa, is well-documented. Another less well-known presentation and target site of this disease is the human breast. Strikingly a high incidence of male breast cancer is found in several countries of sub-Saharan Africa included in the so-called “malaria belt” (19). Because the virus can
directly infect epithelial cells and is associated with some epithelial cancers, a bank of DNA breast cancers was screened to search for the possible presence of EBV in this context. The experiments carried out here, data from which are summarized in Table 1, have shown at least 21% positivity in the samples tested, with PCR findings being generally confirmed by DNA and RNA ISH. This figure may be a low estimate, since very faint positivity observed in a number of cases was scored by us conservatively as negative.

As the exquisitely sensitive PCR was one technique used in our analysis, the possibility that our data might reflect a contamination problem had to be ruled out. With PCR, one main source of contamination is with amplified products obtained from previous experiments. This possibility was virtually eliminated in our work: the experiments carried out in a laboratory where there had been no previous work on EBV; the amplification step was carried out at a different site; and the manipulation of the PCR products was made in a third laboratory. Positive displacement pipettes were used. No DNA, reagents, pipetting instruments, centrifugation buckets, laboratory coats etc., were used in more than one place. Contamination with plasmids, resulting in a positive signal after hybridization with radiolabeled BamHIW fragment in the Bluescript vector was deemed only a remote risk judging by the fact that only a minority of the samples were positive. This factor was essentially eliminated when we could show that the results obtained with a radiolabeled pure PCR product (not in a plasmid) were indistinguishable from those obtained with the recombinant BamHIW probe. Another series of PCR amplifications was made to corroborate the results obtained initially, and to see if a single-copy gene was as detectable as the reiterated ones. There was a good correlation between the two series (Table 1). A single series of PCR amplifications was made in each case.

Another factor that needed to be considered was the possibility of tumor infiltration with lymphocytes. Such could not, however, fully account for our data. For example, at least one of the samples found positive after amplification showed no evidence of lymphocytic infiltration by histopathology. Moreover, on average, only 1 circulating lymphocyte in 10^5-10^6 cells in normal infected individuals is positive for EBV (34). If derived exclusively from the lymphocyte population, this would yield between 100 ng-1 μg of total DNA. As 1 μg of total tumor DNA was used for the PCR amplification experiments, this would require between 1 and 10 positive B cells in 1 μg of material composed entirely of lymphocytes. The small amount of DNA used by us in our experiments virtually excludes the concentration of cells necessary to find statistically one EBV positive lymphocyte, unless there were a particular clustering of EBV-positive lymphocytes in the tumors; however, the histopathology data (Table 1) revealed either an absence (1 sample) or a mild infiltration of tumor (9 samples) by lymphocytes. Some samples among the EBV-negative tumors did have a marked lymphocytic infiltration (9 of 65 of the negatives where information was available) but no EBV was identified by PCR. Furthermore, in controls using blood samples only 10 of the 91 samples examined here were positive by the protocol we adopted, which was designed for a sensitivity that would not detect very low levels of viral DNA.

In situ hybridization studies, carried out to corroborate our findings, as well as to localize the EBV signal, demonstrated the presence of the viral DNA in some tumor cells per se in an appreciable number (12 cases (63%)) of the positive cases. One of the viral regions examined in our analysis, within BamHIW fragments (Fig. 1), is reiterated 7-12 times in the EBV genome, thus providing a good target for detecting the presence of EBV in a sample where a small viral copy number might be expected. The RNA ISH hybridization for EBERs gave positive results in a smaller percentage of cases. Since EBERs are expressed in high copy numbers in most cells latently infected by EB virus (22), this may imply that the EBERs are only occasionally expressed in these tumors. There may also be an alternative explanation for our results, that is, whereas the indirect ISH technique is more sensitive, the direct one has been used in most of our studies to avoid a strong background, particularly with a prolonged incubation period. Thus, low copy numbers of the RNA might not have been detected. Nonetheless, the direct procedure could identify EBV EBERS in the low EBV copy number Namalwa BL cell line. Thus, the question of the levels of EBERS in these tumors remains to be resolved. The interpretation of ISH remains a subjective process, and the fact that, technically, the sections were less than ideal with many cells coming off the slides and because a background problem was often encountered, added to the hazards of interpretation. However, the difference between the sections hybridized with the DNA probe or antisense RNA probe, or, as controls, the vector only or the sense probe,
respectively, was in general, obvious: a common pattern, particulate for the biotin DNA probe (see Fig. 4) and diffuse for the digoxigenin RNA probe (Fig. 5), was observed in the samples interpreted as positive. Moreover, slides found strongly positive by ISH were also strongly positive by PCR, and samples corresponding to a negative PCR and tested by ISH were found negative when read blind.

In this study, there was no clear difference identified in the clinical stage of the disease, the node status, the histological type or the lymphoplasmocytic reaction of the EBV-positive tumors compared with the EBV-negative samples (see Table 1). Nor, after a median follow-up of only 36 months ($P > 0.8$), was there any evidence that the presence of the virus had any effect on disease-free interval. However, as most of the patients are still in remission, longer term follow-up is required to be clear on this point. The fact that not all samples, or all cells in any particular sample, scored positive for EBV could be interpreted in several ways, e.g., (a) that EBV, where detected in breast tumors is an innocent passenger in the tumor, playing no real role in its genesis. The same argument could be made for other tumors associated with this virus. In this regard, it is interesting to note that in NPC patients, where there is almost total correlation between EBV and disease, in some lesions a substantial proportion of tumor cells had no detectable viral genome. In WHO classes II and III, on the other hand, most tumor cells carried multiple copies of EBV (35). Several studies have shown evidence of EBV association in carcinoma of the cervix (36, 37), whereas others have failed to show any association (38, 39), suggestive of sampling differences. It could possibly be the same case with breast cancer studies here; (b) there could be a EBV reactivation in circulating B lymphocytes in the patient, with subsequent infection and passage to the epithelial neoplastic cells. EBV would then be a late event and not play a direct role in the carcinogenesis; (c) alternatively, some EBV negative tumors may have lost the virus, the latter having played a role in the transformation process by a “hit and run” mechanism (40). There is precedence for this notion in in vitro studies in that NPC cells placed in culture lose their viral DNA (41), as do EBV-carrying immortalized epithelial cells (15), with no alteration of morphology. This points to an initiating event for EBV in cellular transformation; and (d) the EBV positive tumors belong to a specific subgroup (ethnic origin, family history, age at diagnosis, ability to respond to therapy, etc), involve a specific strain of the EBV virus, or otherwise represent a specific subclass of susceptible epithelial cells for the virus. In fact, a study published on medullary carcinoma of the breast and infiltrative ductal carcinoma failed to show any association with EBV when 35 samples were tested by PCR (42). Further studies will be required to distinguish among the above alternatives. In particular, it seems pertinent to determine whether common markers can be identified between NPCs and the EBV-positive breast cancers, whether is the case with BL and NPC (43, 44) elevated titers to EBV antigens precede the disease in some patients “at risk” of developing tumors, and whether viral presence (and expression) could in some cases provide a useful prognostic marker for this important pathology.

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