Lack of Association between EBV and Breast Carcinoma

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Abstract

Multiple conflicting findings have been presented which indicate that EBV may be found in anywhere from 0% to 51% of breast carcinomas. When EBV has been found causally associated with other human cancers, its DNA and one or more of its viral products have been detected in most tumor cells of a given biopsy. To test whether EBV has such an association with breast cancer, we measured the number of viral DNA molecules per cell in matched normal and tumor biopsies from 45 patients using real-time quantitative PCR. In no case could EBV DNA consistently be detected, with either of two different probes, at levels above 0.1 molecules per cell in two sections of the tumor samples. These levels of detection match those detected in EBV-negative cell lines and therefore likely represent noise in the assays. Equally importantly, the distribution of these low signals was the same between tumors and their matched normal controls. We conclude that EBV does not contribute to the development of breast cancers as it does to epithelial cancers such as nasopharyngeal and gastric carcinomas or to Burkitt's and Hodgkin's lymphomas. (Cancer Epidemiol Biomarkers Prev 2005;14(4):809–14)

Introduction

EBV, a ubiquitous γ herpesvirus, is a risk factor for developing Burkitt's lymphoma, nasopharyngeal carcinoma, post-transplant lymphoproliferative disorder, a subset of Hodgkin lymphomas, and gastric carcinomas (1). In these cancers, for which epidemiologic and molecular virological data support a causal link, EBV DNA and one or more viral gene products have been detected in the majority of tumor cells (1). Recently, EBV was implicated as a possible contributor to a subset of breast carcinomas (2). The data bearing on this putative association of EBV with breast cancer are, however, contradictory and therefore inconclusive. These contradictions reflect the different assays used, their different sensitivities, and different definitions of ''EBV positive''.

Two general targets have been used in attempts to detect EBV in breast tumor samples: viral products such as the EBV-encoded small RNAs (EBER); small RNAs transcribed by RNA polymerase III; or EBNA1, a nuclear protein essential for maintaining the viral genome and the viral genome DNA itself. The EBERs can be expressed in cells in culture to levels as high as 10^7 copies per cell and when expressed efficiently can be detected confidently by *in situ* hybridization (3, 4). EBNA1 can be detected by immunohistochemistry but its detection is often not robust and can be ''nonspecific'' (5). The detection of EBERs and EBNA1 in breast tumor samples has ranged from 42% being positive for EBNA1 (6) or 25% of samples being positive for EBNA1 with a mostly nonoverlapping 10% being positive for EBERs (7) to no samples being positive for EBNA1 or for EBERs (8-11). The detection of EBV DNA by Southern blotting or PCR methods in breast cancers has ranged from being as high as 21%, 31%, and 51% being positive (12-14) to as low as 6% to 10% of samples being positive (7, 11, 15). The assays for viral DNA are direct and sensitive but have suffered from lack of normalization of the data to the number of cell equivalents being assayed (2). The variation in the results of these different assays is illustrated by contrasting three studies that used detection of viral products finding 0 of 210 cases to be EBV positive (8-10) to seven studies that measured viral DNA and found 262 of 955 cases to be EBV positive (6, 7, 15). This variation indicates that EBV gene products may not be expressed detectably in EBV-positive tumor cells. It further illustrates the need to assay for the presence of EBV DNA in breast cancers quantitatively and to normalize these measurements for the number of cells being assayed.

We define ''EBV positive'' to mean that the majority of tumor cells within a biopsy each have one or more copies of EBV DNA, because this property is found in all human tumors for which EBV is an established, contributing risk factor. We have assayed tumors and matched normal controls by real-time quantitative PCR (RT-QPCR) for the presence of EBV DNA by targeting two regions of the viral genome and have normalized these measurements to the level of cellular DNA measured by RT-QPCR in parallel. This normalization allows a test for tumors being ''EBV positive'' (i.e., having EBV DNA in the majority of tumor cells) or not.

Materials and Methods

Patient Samples. Matched normal and breast carcinoma samples from 45 patients were obtained from the University of Wisconsin-Madison Clinical Hospital. All patients had consented to use of their tissues for research purposes, and the study was approved by the University of Wisconsin Health Sciences Institutional Review Board.

As detailed in Table 1, the case series was representative of current clinical practice. Patient ages ranged from 29 to 83 years with a median of 57 years. Histologically, the tumors fell into four categories: 37 (82%) infiltrating ductal, 4 (9%) infiltrating lobular, 3 (7%) tubular carcinomas, and 1 (2.2%) mucinous carcinoma. According to the modified Bloom Richardson grading guidelines, 13 (29%) carcinomas were

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Table 1. Matched breast tumor and normal samples: histopathologic data and EBV DNA molecules per cell

Patient ID	Age	Diagnosis	Grade	Size (cm)	ER	PR	LNM	% Tumor cells	Section A			Section B		
									β-Actin DNA	EBV DNA/cell: BALF5	EBV DNA/cell: Raji	β-Actin DNA	EBV DNA/cell: BALF5	EBV DNA/cell: Raji
1	65	NML							210	0	0	875	0.058	0
		IDC	3	1.8	+	−	+	33-66	5,714	0	0.0012	6,124	0	0
2	63	NML							63	0	0	79	0	0.0099
		IDC	3	1.2	+	+	+	10-33	198	1.6	0	292	0.043	0.018
3	48	NML							685	0.016	0	1,080	0	0.0017
		ILC	1	3	+	−	+	1-10	1,149	0	0	1,691	0	0.0025
4	59	NML							260	0	0	911	0	0.0019
		IDC	2	1.7	+	−	−	10-33	7,428	0.0071	0.041	5,419	0.0015	0
5	46	NML							63	0	0	158	0.28	0.0046
		IDC	3	1.7	−	−	NA	10-33	2,183	0	0	4,381	0.0064	0.0011
6	65	NML							291	0	0	724	0	0.0035
		IDC	2	4.5	+	−	+	33-66	6,109	0.003	0.0047	10,332	0.011	0.0002
7	50	NML							1,389	0.019	0.038	3,833	0	0.0007
		ILC	2	0.8	+	+	−	33-66	746	0.058	0.31	789	0	0.0004
8	60	NML							9,212	0	0	9,663	0	0.0001
		IDC	1	1	+	+	+	10-33	5,655	0	0	7,718	0.0005	0.0009
9	53	NML							126	0.64	0	322	0	0.0024
		IDC	1	0.4	+	+	−	10-33	1,198	0.028	0.031	2,843	0.2	0
10	64	NML							5,052	0	0	1,420	0	0.0013
		IDC	1	1.4	+	+	NA	10-33	4,359	0.0006	0	1,079	0.11	0.0026
11	45	NML							2,394	0	0	1,264	0	0.0009
		IDC	2	2.5	+	+	+	10-33	13,503	0	0.0002	2,112	0.013	0.0045
12	54	NML							879	0.0008	0	125	0.54	0.017
		IDC	2	1.3	−	−	−	1-10	12,484	0.0022	0	759	0.072	0.0085
13	58	NML							7,631	0.001	0	595	0.016	0.025
		IDC	2	3	+	+	+	33-66	6,470	0	0.0014	2,686	0	0.0022
14	63	NML							2,363	0.0046	0	130	0.25	0.053
		IDC	2	6	+	−	+	>66	24,631	0	0.0003	1,257	0.028	0.065
15	68	NML							361	0	0.012	561	0	0.01
		IDC	1	2.9	+	+	NA	10-33	838	0	0	333	0.0063	0.015
16	66	NML							7,202	0.0051	0	3,525	0.18	0
		IDC	1	1.5	+	+	−	33-66	28,255	0	0	9,952	0	0.0001
17	49	NML							2,096	0	0.0022	559	0.0031	0.02
		IDC	2	4.8	+	−	+	10-33	1,693	0.0003	0	940	0.0051	0
18	57	NML							2,706	0.0063	0	370	0.34	0.0024
		IDC	3	2	−	−	NA	33-66	58,627	0	0.0001	2,665	0.0015	0.0036
19	46	NML							339	0	0.0075	193	0	0.019
		IDC	2	6	+	+	−	10-33	972	0	0.0093	1,256	0	0.0087
20	72	NML							1,189	0.0051	0	102	2.4	0.16
		IDC	1	0.7	+	−	NA	10-33	106	0.1	0	43	0.48	0
21	57	NML							33	0	0	16	2.2	0.054
		TUB	1	0.8	+	−	NA	10-33	3,543	0.018	0	98	0.76	0.11
22	46	NML							1,942	0	0	297	0.17	0.074
		IDC	3	1.5	−	−	NA	1-10	3,409	0.0003	0.0004	950	0	0.0079
23	44	NML							2,300	0.0112	0	412	0.36	0.0035
		IDC	3	4	+	−	−	10-33	7,109	0.0008	0.012	34,731	0	0.0001
24	64	NML							1,465	NA	0.0049	789	0.0014	0.013
		IDC	3	3	−	−	NA	10-33	17,110	0	0	583	0.19	0
25	29	NML							187	0.0099	0.04	352	0	0.021
		IDC	3	1	−	−	NA	33-66	11,092	0.0014	0	813	0.12	0.035
26	35	NML							21,729	0	0	401	0.67	0.57
		IDC	2	5	+	−	+	>66	28,631	0.0003	0	12,347	0.071	0.0005
27	35	NML							2,206	0	0	2,704	0.0014	0.0014
		IDC	3	2	−	−	−	10-33	19,873	0	0.0026	18,863	0	0
28	57	NML							38	0.26	0	202	1.4	NA
		IDC	2	3.5	+	+	+	33-66	958	0.003	0	859	0.16	0
29	70	NML							11	0	0	24	0	0
		ILC	3	3	−	−	NA	>66	254	0.25	0	1,362	0.37	0
30	76	NML							63	0	0	170	0	0
		TUB	1	1.5	+	+	NA	1-10	2,300	0.0008	0	3,574	0.023	0.0009
31	64	NML							54	1.3	0	544	0.3	0.0045
		IDC	2	2.2	+	−	+	10-33	706	0	0	359	0	0
32	76	NML							100	0	0	88	0	0
		IDC	1	1.1	+	−	−	10-33	3,629	0.0015	0.0003	4,228	0	0.0009
33	67	NML							849	0	0	3,154	0.0013	0.0004
		IDC	1	0.9	+	+	−	33-66	45	0	0	888	0	0
34	83	NML							31	0.29	0	29	0	0
		MUC	1	1	+	+	NA	10-33	524	0.0093	0	4,981	0.0004	0.0005
35	78	NML							65	0	0	98	0	0
		IDC	2	2.3	+	+	NA	33-66	4,831	0.0004	0	15,980	0.0001	0
36	71	NML							553	0	0.0018	2,477	0.026	0
		IDC	2	2	+	−	+	33-66	10,970	0	0	9,548	0	0
37	52	NML							228	0.13	0	525	0	0

(*Continued on the following page*)

Table 1. Matched breast tumor and normal samples: histopathologic data and EBV DNA molecules per cell (Cont'd)

Patient ID	Age	Diagnosis	Grade	Size (cm)	ER	PR	LNM	% Tumor cells	Section A			Section B		
									β-Actin DNA	EBV DNA/cell: BALF5	EBV DNA/cell: Raji	β-Actin DNA	EBV DNA/cell: BALF5	EBV DNA/cell: Raji
		TUB	1	0.7	+	−	−	10-33	1,603	0.0014	0.0006	2,330	0.016	0.0004
38	43	NML							27	0	0	13	5.5	0
		ILC	3	0.8	+	+	−	1-10	1,089	0	0	842	0	0
39	50	NML							53	0	0	63	0	0
		IDC	1	0.9	NA	NA	−	10-33	196	0.23	0	619	0	0
40	45	NML							1,055	0	0	498	0	0
		IDC	2	4	+	+	+	33-66	6,981	0	0	5,509	0	0
41	55	NML							112	NA	0	175	0	0
		IDC	2	1.1	+	−	+	1-10	470	0	0	468	0	0
42	44	NML							91	0	0	147	0	0
		IDC	1	1.8	+	+	−	33-66	337	0	0	307	0	0
43	42	NML							495	0	0	350	0	0
		IDC	3	5	−	−	+	>66	3,720	0	0	2,646	0	0
44	56	NML							178	NA	0	194	0	0
		IDC	3	3.5	−	−	−	10-33	1,491	0	0	1,049	0	0
45	67	NML							562	0	0.0025	542	0	0
		IDC	2	2.5	+	+	NA	10-33	3,306	0	0	2,588	0	0
		PTLD							10,181	27	NA	12,499	36	3.5
		PTLD							1,441	109	32	1,313	106	70
		PTLD							11,384	229	74	11,527	312	88
		PTLD							658	88	5	364	37	4
Median	57			1.8										
Minimum	29			0.4										
Maximum	83			6										

Abbreviations: NML, normal; IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; TUB, tubular carcinoma; MUC, mucinous carcinoma; PTLD, post-transplant lymphoproliferative disease; ER, estrogen receptor; PR, progesterone receptor; LNM, lymph node metastasis; NA, not assayed.

classified as grade 3, 17 (38%) as grade 2, and 15 (33%) as grade 1. Tumor sizes varied from 0.4 to 6.0 cm with a median of 1.8 cm. Thirty-four (77%) cases were estrogen receptor positive and 10 (23%) cases were negative. Lymph node sampling data were available from 31 patients: 16 (52%) had lymph node metastases and 15 (48%) were negative.

All samples were formalin-fixed, paraffin-embedded, archived samples. For each patient, four 20-μm sections of tissue (2 tumor and 2 adjacent normal) were obtained for RT-QPCR analysis. An additional 5-mm section was used for H&E staining to determine tumor content and level of lymphocyte infiltration, and when indicated, another section was obtained for *in situ* hybridization to EBERs.

Cell Lines. BJAB, 721, and Raji cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 100 units/mL penicillin and streptomycin. HeLa cells were grown in DMEM and supplemented as above. All cells were cultured at 37°C with maximum humidity and 5% CO_2.

DNA Extraction. Two adjacent 20-μm sections were used to obtain DNA for RT-QPCR analysis. Briefly, samples were dewaxed twice with 1 mL xylene for 10 minutes, washed twice with 100% ethanol, dried and resuspended in 500 μL K buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L $MgCl_2$, 100 μg/mL gelatin, 0.45% IGEPAL, and 0.45% Tween 20]. Proteinase K was added to 100 μg/mL and samples were digested overnight at 55°C. Samples were extracted with phenol and chloroform, ethanol precipitated, and the nucleic acid was dissolved in 100 μL double-distilled water. Five microliters aliquots were used for RT-QPCR.

When extracting DNA from cells in culture, the cells were pelleted and resuspended at 5×10^6 cells per mL in DNA lysis buffer [150 mmol/L NaCl, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8.0), 1% SDS, and 20 μg/mL proteinase K] followed by overnight digestion at 55°C, phenol and chloroform extractions, and ethanol precipitation. The pellet was dissolved in 400 μL TE [10 mmol/L Tris-HCl and 1 mmol/L EDTA (pH 8.0)] with 100 μg/mL RNase A and incubated for 1 hour at 37°C. Phenol/chloroform extractions and ethanol precipitation were repeated and the final DNA pellet was dissolved in 100 μL TE.

Real-time Quantitative PCR. RT-QPCR was used to determine the number of cells in a given tissue section, based on β-actin gene content, as well as the number of molecules of EBV based on two distinct viral probes. Ten nanograms of DNA extracted from EBV-positive and EBV-negative cell lines served as controls and water was used to test for DNA contamination in the reagents. RT-QPCR conditions were as follows: 1× Amplitaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA), 0.5 μmol/L each primer, 0.2 μmol/L probe, 1× ROX reference dye (Invitrogen, Carlsbad, CA), and water to 20 μL. PCR cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. The following oligonucleotide sequences were used for RT-QPCR primers and probes: β-actin, forward primer 5′-TCACCCACACTGTGCCCATCTACGA-3′, reverse primer 5′-TGAGGTAGTCAGTCAGGTCCCG-3′, and probe 5′-ATGCCC TCCCCCATGCCATCCTGCGT-3′; Raji, forward primer 5′-TGACCTACTTGGACCATGTGGA-3′, reverse primer 5′-TGATGAGACTTCCGAGTGCACT-3′, and probe 5′-CAGTGTCCTGATCCTGGACCTTGACTATGAA-3′; BALF 5, forward primer 5′-CGGAAGCCCTCTGGACTTC-3′, reverse primer 5′-CCCTGTTTATCCGATGGAATG-3′, and probe 5′-TGTACACGCACGAGAAATGCGCC-3′.

EBER *In situ* Hybridization. *In situ* hybridization for EBER detection was carried out on selected samples using a commercial kit (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom). Samples were analyzed by microscopy for EBER-stained nuclei. Formalin-fixed, paraffin-embedded sections from EBV-positive nasopharyngeal carcinoma and post-transplant lymphoproliferative disease and the EBV-negative lymphoblastoid cell line BJAB were used as controls.

Statistical Methods. Duplicate measurements were averaged geometrically. The benefit in the context of low signal is that a positive average implies positive in both duplicates. Two such averages were available for each probe in both normal and tumor sections. To test for differences between tumor and normal on a given probe, measurements were first transformed to their ranks among all measurements for that probe; ties were broken by averaging ranks. The rank transformation alleviates potential instability caused by variation over orders of magnitude. For each subject, a difference was calculated between the average of the two normal ranks and the average of the tumor ranks; these differences were accumulated over subjects to obtain a test statistic, which was then calibrated by shuffling ranks within subjects and recomputing the test statistic. This permutation approach accommodates the matched-pair structure and produces an exact significance test (16). All computations were done using the R system for statistical computing (17).

Results

We measured the number of EBV DNA molecules relative to the number of cellular genomes by RT-QPCR in 45 matched tumor and normal breast tissue samples. Primers and Taqman probes were designed for two different, nonrepetitive viral DNA targets: BALF5 and Raji. The Raji probe detects DNA sequences absent in the laboratory strain of EBV (B95.8) but present in clinical isolates such as the Raji strain and thus controls for laboratory contamination. All probes were validated with DNA extracted from two EBV-negative (BJAB and HeLa) and two EBV-positive (Raji and 721) cell lines. As expected, the Raji probe failed to detect EBV DNA in the EBV B95.8-infected 721 cell clone but detected EBV DNA in Raji cells; the BALF5 probe detected EBV DNA in extracts of both cells. Signal derived from the Raji probe ranged between 0.1 and 0.5 of that of the BALF5 probe.

The number of cell genomes or cell equivalents in each sample was measured using RT-QPCR for β-actin, simultaneously insuring that each sample could support detection of amplifiable DNA. The β-actin assays provided an unexpected insight. For many extracts from archived, formalin-fixed, paraffin-embedded biopsies, β-actin sequences could be amplified with primers that yielded a 92-bp product but could not be detected in the same samples using primers that gave rise to a 295-bp product. DNA degradation in the archived samples explains this discrepancy because both primer pairs amplified β-actin DNA in samples prepared freshly from cell lines. We therefore used EBV and β-actin primers yielding 89- to 117-bp products for all measurements.

Each of 45 pairs of tissue biopsies was assessed histologically for the fraction of tumor cells using a section flanking that used to extract DNA (Table 1). These assessments indicated that whereas some tumor samples with few cells and a low fraction of tumor cells had on the order of 10 tumor cells, most tumor samples contained 100s to 1,000s of tumors cells. However, neither EBV probe measured a significant difference in EBV DNA concentration between tumor and normal samples ($P > 0.5$). For the majority of tumor samples tested, for which 10% to 66% of the tissue was comprised of tumor cells, <1% of these cells could have even one copy of EBV DNA (Table 1). Additional computations confirmed the absence of a tumor/normal difference when β-actin normalization was made via regression analysis rather than straight division (data not shown). EBV DNA levels in tumor samples and matched normal samples approximated those in DNA preparations from EBV-negative cell lines, which averaged 0.001 to 0.03 molecules of EBV DNA per cell and thus likely reflect noise in the assays. The occasional outlying, high signals (Fig. 1) may reflect rare EBV-infected cells, including infiltrating lymphocytes that harbor multiple copies of viral DNA.

A second indication that the breast cancer cells generally lacked EBV DNA was that the EBV DNA signals did not increase with β-actin DNA for tumor or matched normal samples, again making it likely that most signals for EBV DNA represented noise in the assay (Fig. 1). The general absence of EBV DNA from breast tumor biopsies led us to test four similarly prepared and stored post-transplant lymphoproliferative disorder samples known to be EBV positive to insure that such fixed, archived samples yielded reasonable levels of signals with all probes (Table 1). These EBV signals generally increased with increasing number of cell equivalents; that is, as the measured levels of β-actin increased in samples, the level of EBV DNA detected increased too, consistent with this detected EBV DNA being true signals (Fig. 1).

Eleven samples that had one or more measurements of >0.01 EBV DNA molecules per cell were tested by EBER *in situ* hybridization. None of these samples had EBER-stained nuclei in tumor or normal epithelial cells nor in any infiltrating lymphocytes of the biopsy section (data not shown). Post-transplant lymphoproliferative disorder samples served as positive controls, whereas formalin-fixed, paraffin-embedded BJAB sections served as a negative control. The absence of detectable EBER staining indicates either that the signals in the RT-QPCR represent noise in the assay, or that rare EBV-positive cells are present in one but not another section of a given sample.

Discussion

All cancers for which EBV is an established risk factor maintain multiple copies of EBV DNA and express one or more viral proteins in most tumor cells (1). We tested whether breast cancers share this association with EBV by measuring viral genome load in tumor and matched normal biopsies using RT-QPCR. Primers and Taqman probes used were validated with DNA isolated from EBV-negative and EBV-positive cell lines. These probes detected ~1 molecule of EBV DNA per 1,000 molecules of cell DNA for most of the 45 tumor and matched normal samples (Table 1). Sections from selected samples with relatively high EBV DNA measurements were also tested for cells expressing EBERs. No EBER-positive cells were detected, indicating either that the RT-QPCR signals reflect noise or that rare EBV-positive cells exist in some of the tumor and normal samples and could be detected in the 20-μm sections used to isolate DNA but not in the 5-μm sections used to detect EBERs. Overall, these data indicate that EBV was no more common in breast carcinoma cells than in matched normal cells and in both cases, it is usually is present in <1% of the cells. EBV was therefore not associated with breast cancer in this patient population. Lack of a significant difference in EBV DNA concentration between tumor and normal tissues does not reflect a lack of statistical power. The situation is well approximated by a one-sided paired t test using $n = 45$ matched samples. The noise level in measurements with both EBV probes is <1.0 molecule per cell allowing detection of average differences on the order of one molecule per cell.

Our consistent findings are not incompatible with earlier studies that were interpreted to indicate that products of EBV and/or its DNA can be detected in a fraction of breast cancers and therefore is associated with this cancer, but suggest reevaluation of those studies. False-positive results

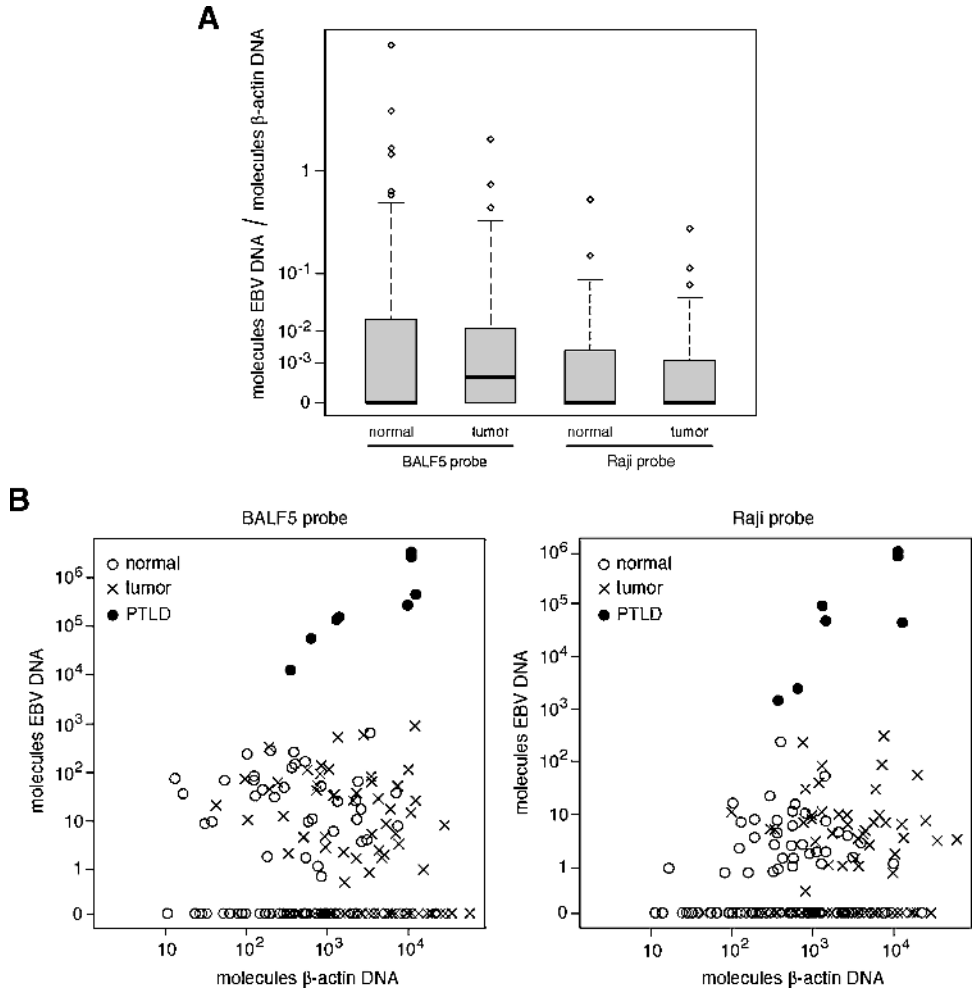

Figure 1. EBV DNA concentration in tumor and normal tissue. For each of two EBV probes (BALF5 and Raji), duplicate measurements in two replicate sections from $n = 45$ tumor samples and $n = 45$ matched normal samples are shown. **A.** Boxplots of the distribution of EBV DNA concentrations (normalized to β-actin). Boxes delineate the interquartile range between the 25th and 75th percentiles; medians (*heavy solid lines*). Dashed lines extend beyond the 75th percentile a distance 1.5 interquartile range; ◇, outlying values (17). For either probe, no statistically significant differences in EBV DNA concentration exist between tumor and normal samples, as determined by permutation tests. Low median values are caused by the large number of zero concentration measurements. **B.** Scatterplots relating geometric averages $[(xy)^{1/2}]$ of EBV DNA concentration to β-actin measurements. *Points at the bottom,* exact zero values in which at least one of the duplicate measurements equals zero. For BALF5 and Raji probes, respectively, 52 and 54 of 90 normal measurements and 44 and 52 of 90 tumor measurements equal 0. Consistent with cell density, tumors have slightly higher β-actin values than normals. PTLD samples are EBV-positive controls.

can clearly arise in attempts to detect EBNA1 because of cross-reactivities of some antibodies thought to be ''specific'' for EBNA1 (5). False-positive results in detection of EBERs can arise from the *in situ* hybridizations to detect it not being quantitative; low levels of expression of EBERs are distinguishable from background staining only subjectively. There are also difficulties in methods use to detect viral DNA. For example, detection of viral DNA in breast cancers has largely depended on the use of PCR techniques with 35 to 50 cycles of amplification often followed by a second round of amplification or in combination with detection by Southern blotting (7, 11-14). In theory, these methods could detect a single molecule of EBV DNA and thus do not necessarily associate EBV DNA with the tumor cells present in a biopsy. In one instance, microdissected tumor samples with only one EBV DNA molecule per 1,000 cell equivalents have been classified as ''EBV positive'' (14), whereas in another, samples positive for EBV DNA by PCR but negative by the less sensitive Southern blotting were considered not significant (7). The extreme sensitivity of PCR-mediated detection of DNA, coupled with the varying definitions of ''EBV-positive'' have led to conflicting interpretations of EBV's potential association with breast cancer. In addition, where EBV DNA was detected more often in tumor than normal tissue, the increased cellularity of tumor relative to normal samples could have favored detection of occasional EBV-infected cells in tumor samples over normal samples. The sporadic presence of such infected cells could not be appreciated without normalizing the level of viral DNA detected to the total cellular DNA assayed. It is also evident that rare breast cancers may contain many cells infected with EBV. However, we did not encounter such exceptions in these 45 cases.

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References

1. Crawford DH. Biology and disease associations of Epstein-Barr virus. Philos Trans R Soc Lond B Biol Sci 2001;356:461–73.
2. Glaser SL, Hsu JL, Gulley ML. Epstein-Barr virus and breast cancer: state of the evidence for viral carcinogenesis. Cancer Epidemiol Biomarkers Prev 2004;13:688–97.
3. Howe JG, Steitz JA. Localization of Epstein-Barr virus-encoded small RNAs by *in situ* hybridization. Proc Natl Acad Sci U S A 1986;83:9006–10.
4. Ambinder RF, Mann RB. Epstein-Barr-encoded RNA *in situ* hybridization: diagnostic applications. Hum Pathol 1994;25:602–5.
5. Murray PG, Lissauer D, Junying J, et al. Reactivity with a monoclonal antibody to Epstein-Barr virus (EBV) nuclear antigen 1 defines a subset of aggressive breast cancers in the absence of the EBV genome. Cancer Res 2003;63:2338–43.
6. Grinstein S, Preciado MV, Gattuso P, et al. Demonstration of Epstein-Barr virus in carcinomas of various sites. Cancer Res 2002;62:4876–8.
7. Chu PG, Chang KL, Chen YY, Chen WG, Weiss LM. No significant association of Epstein-Barr virus infection with invasive breast carcinoma. Am J Pathol 2001;159:571–8.
8. Glaser SL, Ambinder RF, DiGiuseppe JA, Horn-Ross PL, Hsu JL. Absence of Epstein-Barr virus EBER-1 transcripts in an epidemiologically diverse group of breast cancers. Int J Cancer 1998;75:555–8.
9. Chu JS, Chen CC, Chang KJ. *In situ* detection of Epstein-Barr virus in breast cancer. Cancer Lett 1998;124:53–7.

10. Deshpande CG, Badve S, Kidwai N, Longnecker R. Lack of expression of the Epstein-Barr Virus (EBV) gene products, EBERs, EBNA1, LMP1, and LMP2A, in breast cancer cells. Lab Invest 2002;82:1193–9.
11. Herrmann K, Niedobitek G. Lack of evidence for an association of Epstein-Barr virus infection with breast carcinoma. Breast Cancer Res 2003;5:R13–7.
12. Labrecque LG, Barnes DM, Fentiman IS, Griffin BE. Epstein-Barr virus in epithelial cell tumors: a breast cancer study. Cancer Res 1995;55:39–45.
13. Bonnet M, Guinebretiere JM, Kremmer E, et al. Detection of Epstein-Barr virus in invasive breast cancers. J Natl Cancer Inst 1999;91:1376–81.
14. Fina F, Romain S, Ouafik L, et al. Frequency and genome load of Epstein-Barr virus in 509 breast cancers from different geographical areas. Br J Cancer 2001;84:783–90.
15. McCall SA, Lichy JH, Bijwaard KE, Aguilera NS, Chu WS, Taubenberger JK. Epstein-Barr virus detection in ductal carcinoma of the breast. J Natl Cancer Inst 2001;93:148–50.
16. Lehman EL. Testing statistical hypotheses. New York: Springer-Verlag; 2002.
17. R Development Core Team (2004). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-00-3, URL http://www.R-project.org.