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Epstein-Barr Virus (EBV) Genome and Expression in Breast Cancer Tissue: Effect of EBV Infection of Breast Cancer Cells on Resistance to Paclitaxel (Taxol)

Hratch Arbach,[1] Viktor Viglasky,[1]† Florence Lefeu,[1] Jean-Marc Guinebretière,[2] Vanessa Ramirez,[1] Nadège Bride,[1] Nadia Boualaga,[1] Thomas Bauchet,[1] Jean-Philippe Peyrat,[3] Marie-Christine Mathieu,[5] Samia Mourah,[1] Marie-Pierre Podgorniak,[1] Jean-Marie Seignerin,[4] Kenzo Takada,[6] and Irène Joab[1]*

INSERM U716, IUH, IFR Saint-Louis, 27 rue Juliette Dodu, 75010 Paris, France[1]; Centre René Huguenin, Service de Pathologie, 35 rue Dailly, Saint-Cloud, France[2]; Centre Oscar Lambret, laboratoire d'Oncologie Moléculaire Humaine, Lille, France[3]; Faculté de Médecine de Grenoble, Laboratoire de Virologie Médicale Moléculaire, Domaine de la Merci, La Tronche, France[4]; Institut Gustave Roussy, Villejuif, France[5]; and Department of Tumor Virology, Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan[6]

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The Epstein-Barr virus (EBV) has been detected in subsets of breast cancers. In order to elaborate on these observations, we quantified by real-time PCR (Q-PCR) the EBV genome in biopsy specimens of breast cancer tissue as well as in tumor cells isolated by microdissection. Our findings show that EBV genomes can be detected by Q-PCR in about half of tumor specimens, usually in low copy numbers. However, we also found that the viral load is highly variable from tumor to tumor. Moreover, EBV genomes are heterogeneously distributed in morphologically identical tumor cells, with some clusters of isolated tumor cells containing relatively high genome numbers while other tumor cells isolated from the same specimen may be negative for EBV DNA. Using reverse transcription-PCR, we detected EBV gene transcripts: EBNA-1 in almost all of the EBV-positive tumors and RNA of the EBV oncoprotein LMP-1 in a smaller subset of the tissues analyzed. Moreover, BARF-1 RNA was detected in half of the cases studied. Furthermore, we observed that in vitro EBV infection of breast carcinoma cells confers resistance to paclitaxel (taxol) and provokes overexpression of a multidrug resistance gene (MDR1). Consequently, even if a small number of breast cancer cells are EBV infected, the impact of EBV infection on the efficiency of anticancer treatment might be of importance.

The Epstein-Barr virus (EBV), a ubiquitous human herpesvirus, is associated with the development of different epithelial malignancies (28), including nasopharyngeal carcinoma (NPC). It has also been linked with subsets of other types of carcinomas, including gastric carcinoma and lymphoepithelioma-like carcinoma in salivary glands and thymus (32, 44). Several laboratories have reported detection of EBV in a subset of breast tumors (3, 10, 19, 23). However, negative results have also been reported (6, 9, 14, 27). Nevertheless, in most of the studies, a low viral load was detected in breast cancer biopsy specimens but the infected cells were not clearly identified. In the study presented here, we used real-time PCR (Q-PCR) to quantify the copy numbers of the EBV genome in biopsy specimens as well as in microdissected tumor cells. The results show that breast cancer cells harbor the viral genome. However, through microdissection and isolation of pure tumor cells, we now find that even in EBV-positive tumor samples, many tumor cells do not contain EBV genomes and that the breast carcinomas are highly heterogeneous in terms of genome content and distribution. Moreover, using reverse transcription-PCR (RT-PCR), we detected EBNA-1 and BARF-1 transcripts in almost all of the EBV-positive tumors and LMP-1 RNA in 3 of the 15 cases studied. The findings raise the possibility that although EBV is unlikely to have an etiologic role in the genesis of breast cancer, the virus might contribute to tumor progression. Finally, the potential impact of EBV in breast cancer progression was evaluated by estimation of resistance to chemotherapeutic agents on in vitro-infected MDA-MB-231 cells. The results show that EBV confers paclitaxel (taxol) resistance and causes overexpression of a multidrug resistance gene (MDR1). Consequently, if even a small number of breast cancer cells are EBV infected, the impact of EBV infection on the efficiency of anticancer treatment might be of importance.

MATERIALS AND METHODS

Biopsy specimens. This study includes breast cancer specimens from 37 patients from Institut Gustave Roussy (Villejuif, France) and 95 from the Centre Oscar Lambret (Lille, France). Tumor specimens were obtained with the agreement of the patients and were solely adenocarcinomas. At the time of collection, fat was removed and samples were divided into three parts. The first part was submitted for histological study and histoprognostic grading according to the method of Contesso et al. (8). The second part of the sample was immediately frozen in liquid nitrogen for estrogen receptor (ER) and progesterone receptor (PR) assays (2). The third part was used in molecular biologic assays (30).

The median age of the patients was 58 years (range, 26 to 90 years). Node involvement was detected in 58.3% of the cases. The tumor size was <1 cm in diameter in 2.3% of the cases, >1 and <3 cm in 58.6% of cases, and >3 cm in 39.1% of cases. The tumor type was ductal in 58.6% of the cases, lobular in 11.5% of the cases, and of other types in 29.8% of the cases. The distribution of histoprognostic grading was 8.4% for type I, 41% for type II, and 50.6% for type III (8); 66% of the tumors were ER positive, and 64% were PR positive.

* Corresponding author. Mailing address: INSERM U716, IUH, IFR Saint-Louis, 27 rue Juliette Dodu, 75010 Paris, France. Phone: 33 (1) 42 49 92 68. Fax: 33 (1) 42 49 48 38. E-mail: Irene.Joab@stlouis.inserm.fr.

† Present address: Institute of Chemistry, Faculty of Sciences, Koöice, Slovak Republic.

TABLE 1. Oligonucleotide sequences

Target	Primer	Primer sequence (5′→3′)	B95-8 genomic coordinates (nucleotides)
EBNA-1	E1AS	TTGCAGCCAATGCAACTTGG	108197–108178
	E1S	AGAGAGTAGTCTCAGGGCAT	67545–67564
BamH1U	UPUS	GTTCCTCGGTGGCGGGCTTA	67378–67397
ExonUAS	U172AS	ATGCCCTGAGACTACTCTCT	67564–67545
LMP-1	LMP1S	CTGAGGATGGAACACGACCT	169480–169461
	LMP1AS	AATGGAGGGAGAGTCAGTCA	168099–168108
BARF-1	BARF1S	GGCTGTCACCGCTTTCTTGG	165560–165579
	BARF1AS	AGGTGTTGGCACTTCTGTGG	165762–165743
BamH1A	BamAS	CGTGGTGAAGCCTCTAACGC	165883–165902
	BamAAS	GGCAAGTGCGTTTATTGCGA	166180–166161
BZLF1	ZS	TTACACCTGACCCATACCAG	103118–103099
	ZAS1	ACATCTGCTTCAACAGGAGG	102284–102303
HPRT	HPRTS	TATGGACAGGACTGAACGTC	
	HPRTAS	GTTGAGAGATCATCTCCACC	

Control DNA from two biopsy specimens of nasopharyngeal carcinoma and five pellets of peripheral blood cells was taken from the study described by Grunewald et al. (12).

Laser capture microdissection. Six frozen breast cancer biopsy specimens collected at Institut Gustave Roussy were taken for microdissection. In each case, one tissue block of breast tumor was cut on a cryostat microtome into 10-μm sections. These were mounted on uncoated slides and fixed immediately in 70% ethanol for 30 s, stained with Mayer's hematoxylin, and then washed in 70%, 90%, and 95% ethanol for 30 s each. Staining was followed by two 60-s dehydration steps in xylene before the samples were air dried. The sections were then microdissected in order to select the tumor cell component. Four samples were dissected with the PixCell II laser capture microdissection (LCM) Arcturus system (Mountain View, California), and two samples were dissected with a Laser Leica LMD. For each tumor block, three or more separate microdissections were taken from different anatomic areas but with no discernible difference in lymphocytic infiltration, cell density, and stromal composition. A minimum of 5,000 cells was captured. The number of cells was evaluated according to the manufacturers' instructions: the number of pulses (Arcturus) or the measurement of the cell surface areas (Leica). Cell populations were estimated to be homogeneous by microscopic visualization. DNA was extracted and subsequently used for Q-PCR.

DNA preparation and Q-PCR. DNA from pulverized frozen specimens or microdissected cells was extracted with a QIAMP mini kit (QIAGEN, Courtaboeuf, France) according to the manufacturer's instructions. Q-PCR of the EBV thymidine kinase gene (BXLF-1) was performed as described by Brengel-Pesce et al. (4) with omission of uracil DNA glycosylase. Dilutions of DNA extracted from Namalwa cells, containing two integrated copies of EBV genomes per cell (21), were used as the external standard. Samples were measured in duplicate. Amplifications of the EBV genome were performed with the number of cycles ranging from 25 to 38. The calibration curve allowed the establishment of the following equation for the number of EBV genomes: $y = -3.320x + 41.50$, with an R^2 value of 0.99 and an error of 0.079. Genomic DNA was quantified by amplification of the β-globin gene. Standardization was performed with DNA from the LightCycler control kit (Roche Molecular Diagnostic, Meylan, France). The calibration curve allowed the establishment of the following equation: $y = -3.312x + 30.06$, with an R^2 value of 1 and an error of 0.0941.

Preparation of RNA, RT-PCR, and Southern blot hybridization. Frozen specimens were pulverized, and total RNA was isolated from frozen tumor tissues, using TRIzol reagent (Gibco-BRL, Gaithersburg, MD) according to the manufacturer's instructions. After treatment of 10 μg of extracted RNA with RQ1 RNase-free DNase (Promega Corp., Madison, WI), cDNA was synthesized from 1 μg of treated RNA using Moloney murine leukemia virus reverse transcriptase (Promega Corp., Madison, WI). PCR was carried out with the cDNA samples obtained from 33 ng of total RNA in a final volume of 50 μl, containing 2 μl of first-strand cDNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 200 nM concentrations of each primer (Table 1), and 250 μM deoxynucleoside triphosphates. Reactions were incubated at 95°C for 5 min, and 3 U of *Taq* DNA polymerase was added to each tube. Forty cycles of cDNA amplification were performed in a DNA thermal cycler (Perkin-Elmer Corp., Foster City, CA) with the following conditions: denaturing at 95°C for 30 s, hybridization at 58°C (55°C for LMP-1 amplification) for 1 min, and elongation at 72°C for 2 min, followed by a final elongation at 72°C for 7 min. Ten microliters of each PCR product was analyzed by electrophoresis in 2% agarose gel and transferred to nylon membranes by Southern blotting (Hybond; Amersham Life Science Inc., Arlington Heights, IL). The blots were prehybridized at 65°C for 2 h with 100 μg/ml of denatured salmon sperm DNA in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5% sodium dodecyl sulfate (SDS), and 5× Denhardt's buffer. Hybridization was performed with an LMP-1 or EBNA-1 cDNA that was labeled with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I (10^6 cpm/ml). Following overnight incubation, membranes were washed twice for 15 min with 2× SSC–0.1% SDS at room temperature, twice with 2× SSC–0.1% SDS at 65°C for 15 min, and twice with 0.1× SSC–0.5% SDS at 65°C for 30 min. The blots were then exposed to X-ray film for imaging.

Cell culture and infection. MDA-MB-231 cells (a cell line derived from pleural effusions of a breast cancer patient.) (5) were grown in RPMI 1640 (Life Technologies, GIBCO BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum and antibiotics at 37°C in a 5% CO_2 humidified atmosphere. The Akata cell clone infected with recombinant EBV (rEBV) was maintained in RPMI 1640 containing 10% fetal bovine serum and G418 (700 mg/ml; Invitrogen, The Netherlands). MDA-MB-231 cells were infected by cocultivation with Akata cells producing rEBV following induction by anti-human immunoglobulin G, as previously described (16). Infected clones were selected after cultivation with G418, and four clones were studied: C1D12, C2G6, C3B4, and C4A3.

Evaluation of cell survival. Three thousand MDA-MB-231 cells attached in a microplate well were subjected to paclitaxel (Sigma). After 3 days, the cell number was estimated by means of the endogenous enzyme hexosaminidase (20).

MDR1 expression. Evaluation of the expression levels of MDR1 (normalized to the housekeeping β2-microglobulin gene) was performed by real-time quantitative PCR using a LightCycler (Roche Diagnostics, France) as described previously (24). β2-Microglobulin and MDR1 selected sets of primers and fluorescent probe (Eurogentec, Biosense, Italy) are the following: b2m forward, 5′CGC TCC GTG GCC TTA GC 3′; b2m reverse, 5′ GAG TAC GCT GGA TAG CCT CCA 3′; b2m probe, 5′ 6-carboxyfluorescein (FAM) TGC TCG CGC TAC TCT CTC TTT CTG GC 3′ 6-carboxytetramethylrhodamine (TAMRA); MDR1 forward, 5′ TGA TGA CCC TAA AAA CAC CAC TG 3′; MDR1 reverse, 5′ GAA CCT ATA GCC CCT TTA ACT TGA 3′; MDR1 probe, 5′ FAM AGC ATT GAC TAC CAG GCT CGC CAA 3′ TAMRA. Standards for MDR1 and β2-microglobulin were prepared from total normal RNA, amplified by RT-PCR, and cloned using a TOPO II TA cloning kit (Invitrogen, France) following the manufacturer's recommendations.

RESULTS

Viral load in breast cancer tissue specimens. Q-PCR assays were performed on 95 whole samples of the breast cancer biopsy tissues. In 51 cases, no copies of the EBV genome were amplified or the number was below the threshold of detection. Forty-four samples were considered positive for EBV, since 2 to 6,000 copies were detected in the assay. Table 2 shows the number of copies of the EBV genome detected in each of the 44 EBV-positive tumor samples. The copy number per 1,000 total cells is determined assuming that one cell contains 6 pg of DNA. The number of EBV genomes was very low; we measured in 9 samples less than 0.1 copy per 1,000 total cells, in 18 samples 0.1 to 0.8 copy per 1,000 total cells, and in 14 samples 1 to 19 copies per 1,000 cells. In two samples, an average of 700 copies was detected, corresponding to 303 and 338 copies in 1,000 total cells. In one sample, exhibiting the highest load, 883 copies per 1,000 cells were measured. In two NPC biopsy specimens, 1.8 and 6 million copies of the EBV genome were detected, corresponding to 27 and 25 copies per cell, respectively (Table 2). The copy number of the EBV genome in breast cancer biopsy specimens is thus very low compared to the ones observed in NPC; however, the EBV genomes were detected, and specimens were considered positive compared to the results obtained in five pellets of peripheral blood lymphocytes from healthy donors, in which the level of EBV DNA was below the threshold of detection.

TABLE 2. Detection of the EBV genome by Q-PCR in biopsied EBV-positive breast cancer tissues

Case[d]	DNA (ng)[a]	Cell equivalents[b]	No. of EBV genomes[c]	Copies of EBV/1,000 cells
BK71	1,606	267,667	4	0.02
BK66	2,386	397,667	6	0.02
BK67	2,505	417,500	8	0.02
BK34	1,096	182,667	5	0.03
BK88	612	102,000	3	0.03
BK69	967	161,166	6	0.04
BK72	890	148,334	6	0.04
BK61	921	153,500	7	0.05
BK62	318	53,000	4	0.08
BK47	188	31,334	3	0.10
BK56	314	52,334	6	0.11
BK70	1,651	275,166	30	0.11
BK39	181	30,166	4	0.13
BK49	291	48,500	7	0.14
BK37	193	32,166	6	0.19
BK6	379	63,166	16	0.25
BK25	134	22,334	7	0.31
BK95	84	14,000	5	0.38
BK83	48	8,000	3	0.38
BK15	141	23,500	9	0.38
BK38	31	5,166	2	0.39
BK24	94	15,667	8	0.51
BK7	248	41,334	22	0.53
BK16	76	12,667	7	0.55
BK12	17	2,833	2	0.70
BK9	73	12,166	10	0.83
BK65	43	7,166	6	0.84
BK21	36	6,000	6	1
BK51	1,277	212,833	214	1
BK68	20	3,334	4	1.2
BK13	93	15,500	23	1.5
BK22	83	13,833	22	1.6
BK30	79	13,166	23	1.8
BK27	363	60,500	118	2
BK40	208	34,667	93	2.7
BK42	12	2,000	7	3.5
BK17	6	1,000	4	4
BK4	163	27,166	214	8
BK8	14	2,334	20	9
BK32	47	7,833	93	12
BK3	54	9,000	170	19
BK2	14	2,334	708	303
BK5	12	2,000	676	338
BK23	47	7,833	6,918	883
NPC1	1,315	219,166	6×10^6	27,376
NPC2	425	70,833	1.8×10^6	25,400
PBLKu15	970	161,667	0	0
PBLKu27	6,940	1.16×10^6	0	0
PBLhk35	17,350	2.9×10^6	0	0
PBLhk39	2,150	358,334	0	0
PBLiKa49	6,100	106	0	0

[a] Quantified by amplification of the ß-globin gene.
[b] Based on the assumption that one cell contains 6 pg of DNA and that there are two copies of the globin gene per cell.
[c] Expressed as the mean of the results of two assays.
[d] BK corresponds to breast cancer biopsies, NPC to nasopharyngeal carcinoma, and PBL to peripheral blood lymphocytes.

Detection and quantification of the EBV genome in isolated malignant epithelial cells of breast tumor biopsy specimens. In order to evaluate the relevance of a low number of viral genomes and to identify the EBV-infected cells within the breast cancer biopsy specimens, we determined the EBV DNA load in isolated cancer cell populations. Six breast cancers from Institut Gustave Roussy were analyzed. Four of them were considered EBV positive, since 4, 11, 2, and 5 copies of the EBV genome were detected in a Q-PCR assay in tumor numbers 240, 393, 200, and 275, respectively. Tumor cells were separated from stromal tissue and normal breast cells by laser capture microdissection (LCM). Figure 1A shows a representative target tissue sample before laser dissection, and Fig. 1B shows the malignant epithelial cells captured after LCM. DNA from two to four different areas containing only carcinomatous epithelial cells was isolated, and the viral load was quantified by Q-PCR. No EBV genomes were detected in three different microdissections taken from two of the six tumors (data not shown). The results obtained with the four EBV-positive tumors are summarized in Table 3. In the microdissected specimens, although the tumor cell population appeared to be homogeneous, only one of four clusters of tumor cells taken from tumor 240 contained EBV genomes (100 copies in 1,000 cells). Similarly, in tumor 393, one of the two studied regions was EBV positive (855 copies in 1,000 cells). All studied regions of the other two tumors (200 and 275) were positive, exhibiting viral loads from 15 to 465 and from 49 to 6,333 copies in 1,000 cells, respectively, showing again a high heterogeneity in viral load. The EBV genome was clearly detected in malignant epithelial areas taken from the four EBV-positive biopsy specimens, demonstrating that the virus is harbored by epithelial cells and not accounted for by infiltrating lymphocytes.

We compared the average viral load detected in the whole tumor specimen with that determined for different microdissected clusters of tumor cells. As expected from the heterogeneity of viral distribution in the tumor cells, the viral load determined within the entire tumor specimen is much lower than that found within a virus-positive region. We determined that in tumor 393, 0.02 copies of the EBV genome were found per 1,000 total cells, although one microdissected region contained 855 copies per 1,000 cells. This result shows that even if a very low copy number of EBV genomes is detected within a whole biopsy specimen, a high copy number can be present in different parts of the tumor.

Detection of EBV transcripts in breast carcinoma biopsy specimens. Tumors harboring the highest viral load were chosen for investigation of EBV expression if a sufficient amount of tissue was available. EBNA-1, BARF-1, and LMP-1 RNA expression was studied in whole tumor specimens. We tested for EBNA-1 transcripts by RT-PCR in RNAs from 21 EBV-positive tumors using E1S and E1AS primers (Table 1) and detected EBNA-1 RNA in 17 of the specimens. An amplified band of the expected size (361 bp) hybridized with labeled specific probe in each case. Typical examples are shown in Fig. 2Ba. Tumors 13, 14, 17, 18, 19, and 21 were positive for EBNA-1 cDNA, while 15 and 20 were negative. Amplification of hypoxanthine-guanine phosphoribosyl transferase (HPRT) cDNA was carried out in each case to control for the quality of the cDNAs (36) (data not shown). These results show that an EBV gene is expressed and detected in almost all of the EBV-positive breast tumors.

In order to detect LMP-1 transcripts, we used primers LMP1S and LMP1AS (Table 1 and Fig. 2Ab). Amplification was performed with cDNA obtained from breast carcinoma biopsy specimens. Three of fifteen samples were positive for

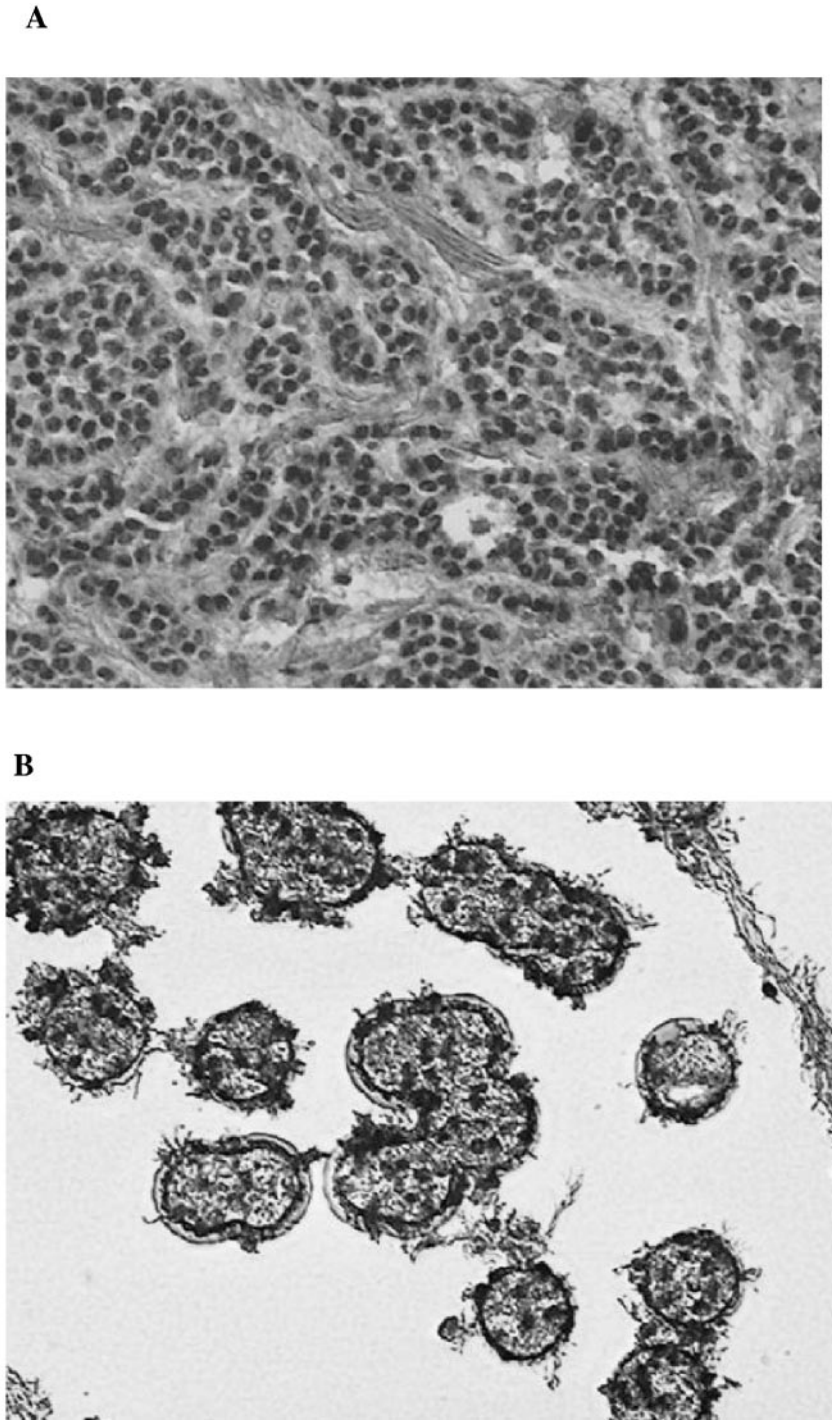

FIG. 1. Laser capture microdissection (LCM) of breast cancer tissue. Frozen tumor sections (10 μm thick) stained with Mayer's hematoxylin and eosin Y were microdissected with the Arcturus PixCell II system to procure homogeneous cell populations. Panel A shows a section before LCM. Panel B shows clusters of the malignant epithelial cells captured by LCM. These are representative sections from the total microdissections summarized in Table 3.

LMP-1 cDNA amplification, as demonstrated by amplification and specific hybridization of a 377-bp band (Fig. 2Bb).

Expression of the EBV BARF-1 gene was investigated by RT-PCR in 14 different breast cancer biopsy specimens using BARF1S and BARF1AS primers. Figure 2Bc shows that a band of the expected size (202 bp) was amplified from cDNAs of 8 of the 14 biopsy specimens. In order to be sure that the bands obtained correspond to copies of cDNA and not to DNA contamination, PCR of untranscribed regions was performed with combinations of primers lying in the U and A

TABLE 3. Samples in which the EBV genome was detected by Q-PCR in whole tumors and microdissected tumor epithelial cells

Biopsy no.	DNA (ng)[a]	No. of cell equivalents[b]	No. of EBV genomes[c]	Copies/ 1,000 cells
240	0.03	5	0	0
	0.035	5	0	0
	0.05	8	0	0
	0.08	13	13	100
393	0.015	2.5	0	0
	0.015	2.5	2	855
200	4	667	10	15
	1.5	250	20	80
	3.9	650	54	83
	0.26	43	20	465
275	0.07	12	76	6,333
	1.2	200	28	140
	1	167	12	72
	1.6	267	13	49

[a] Quantified by amplification of the β-globin gene.
[b] Based on the assumption that one cell contains 6 pg of DNA and that there are two copies of the globin gene per cell.
[c] Expressed as the mean of the results of two assays.

fragments, one primer upstream (UPUS) of the U172 exon and one primer within the U172 exon (U172AS), and also with the combination of one primer (BamAAS) downstream of the BARF-1 sequence and one within the BARF-1 open reading frame (BamAS). No band was obtained with these different cDNA preparations, whereas bands of the expected sizes (185 bp and 317 bp, respectively) were detected after amplification of DNA extracted from B95-8 cells, showing that no genomic DNA was detectable in the cDNA preparations.

BZLF1 expression was assayed in two EBV-negative (986 and BK20) biopsy specimens, eight breast cancer biopsy specimens which harbored the highest viral load for which enough material was available (BK8, BK32, BK3, BK17, BK20, BK9, BK40, and BK23, which contained 9, 12, 19, 4, 0.83, 2.7, and 883 EBV copies/1,000 cells, respectively) (Table 2), and 275 specimens that carried EBV in four of four clusters of tumor cells isolated by LCM (Table 3). RT-PCR followed by Southern blotting was performed. In two tumors, BK3 and BK40, BZLF1 expression was observed after different overexposure of the blot. The pattern of expression is similar to what was seen in NPC (7) and in breast tissues (15).

Paclitaxel resistance of in vitro EBV-infected MDA-MB-231 cells. The potential impact of EBV infection on breast carcinoma cells was investigated. The sensitivity of MDA-MB-231 cells, infected or not infected by EBV, to an agent used in chemotherapy was tested. The cells were subjected to treatment of 3 days of different concentrations of paclitaxel (1, 10, and 100 nM and 1 μM). A representative experiment is shown in Fig. 3. When cultured with 100 nM and 1 μM paclitaxel, 40% and 23% of the noninfected cells survived. One of the infected clones, C4A3, was highly resistant to paclitaxel, since 83% of the cells survived when cultured with 100 nM paclitaxel and 79% survived in 1 μM paclitaxel. Another clone, C2G6, exhibited a resistance, since 60% and 40% of the cells survived in 100 nM and 1 μM paclitaxel, respectively. Several potential mechanisms have been proposed to account for the resistance to paclitaxel (for a review, see Orr et al. [29a]); these include overexpression of the multidrug resistance gene (MDR1). We explored the expression of the MRD1 gene in MDA-MB-231 cells as well as in the infected clones. The results are shown in Fig. 4. The copy number of the MDR1 gene transcripts (normalized to the housekeeping β2-microglobulin gene) was sixfold higher in the C2G6 cell line than in the parental noninfected MDA-MB-231 cell line. The levels of MDR1 expression in the other infected clones were not dramatically increased compared to the ones of the noninfected one. However, when cultured in the presence of 100 nM paclitaxel, the level of MDR1 transcripts was 10-fold higher in the C4A3 clone than the level expressed without paclitaxel in the cultured medium. In summary, in the presence of 100 nM paclitaxel, the level of MDR1 transcripts normalized to β2-microglobulin expression is 6- to 10-fold higher in C4A3 and C2G6 EBV-infected clones than in the parental MDA-MB-231 noninfected clone. C4A3 and C2G6 are more resistant to paclitaxel than the two other infected clones, C1D12 and C3B4. The resistance is correlated with overexpression of the MDR1 gene. The mechanism conferring paclitaxel resistance implicated in EBV-infected MDA-MB-231 cells is currently under investigation.

DISCUSSION

Although a part of the literature showed negative results, EBV has been detected in large subsets of infiltrating breast cancer (3, 10, 19, 23). In this study, we detected EBV genomes in various copy numbers in about half of the breast cancer specimens analyzed. We accounted for this variability by microdissection of the specimens, which disclosed that the distribution of EBV genomes in tumor cells was variable within each tumor specimen even though morphologically the tumor cells were indistinguishable. This observation may account for the variable distribution of the EBNA-1 protein detected by immunohistochemistry (3). It is well known that different biological markers (ER and/or PR, epidermal growth factor receptor, and MMP-2) (35, 37, 38) are heterogeneously distributed within breast cancer tissue. Moreover, Glockner et al. determined the amplification status of growth regulatory genes in macroscopically and microscopically separate areas of individual breast cancers and showed marked intratumoral heterogeneity of c-*myc* and cyclinD1 (11). Intratumoral heterogeneity mirrors subclonal diversity and might affect treatment response. It appears that EBV genomes are also heterogeneously distributed. It would be of interest to identify tissue markers that may correlate with EBV genome content in breast cancer cells that appear otherwise to be morphologically identical. Our results point to heterogeneity in terms of EBV content in populations of breast cancer cells that may be biologically significant.

In different studies, evaluation of EBV in breast cancer has disclosed very low viral loads (10, 27) which have even been defined as EBV negative (31). Murray et al. (27) used 19 sections of paraffin-embedded breast cancer tissues. They measured 2, 12, and 5 samples, respectively, with less than 0.1, 0.1 to 0.9, and 1 to 7 copies of the EBV genome per 1,000 cells. We had similar results but also detected two samples with 303 to 338 copies per 1,000 cells. Moreover, one sample with a load of 883 copies in 1,000 cells was identified among the 95 frozen biopsy tissues. Similarly, one cluster of isolated tumor cells

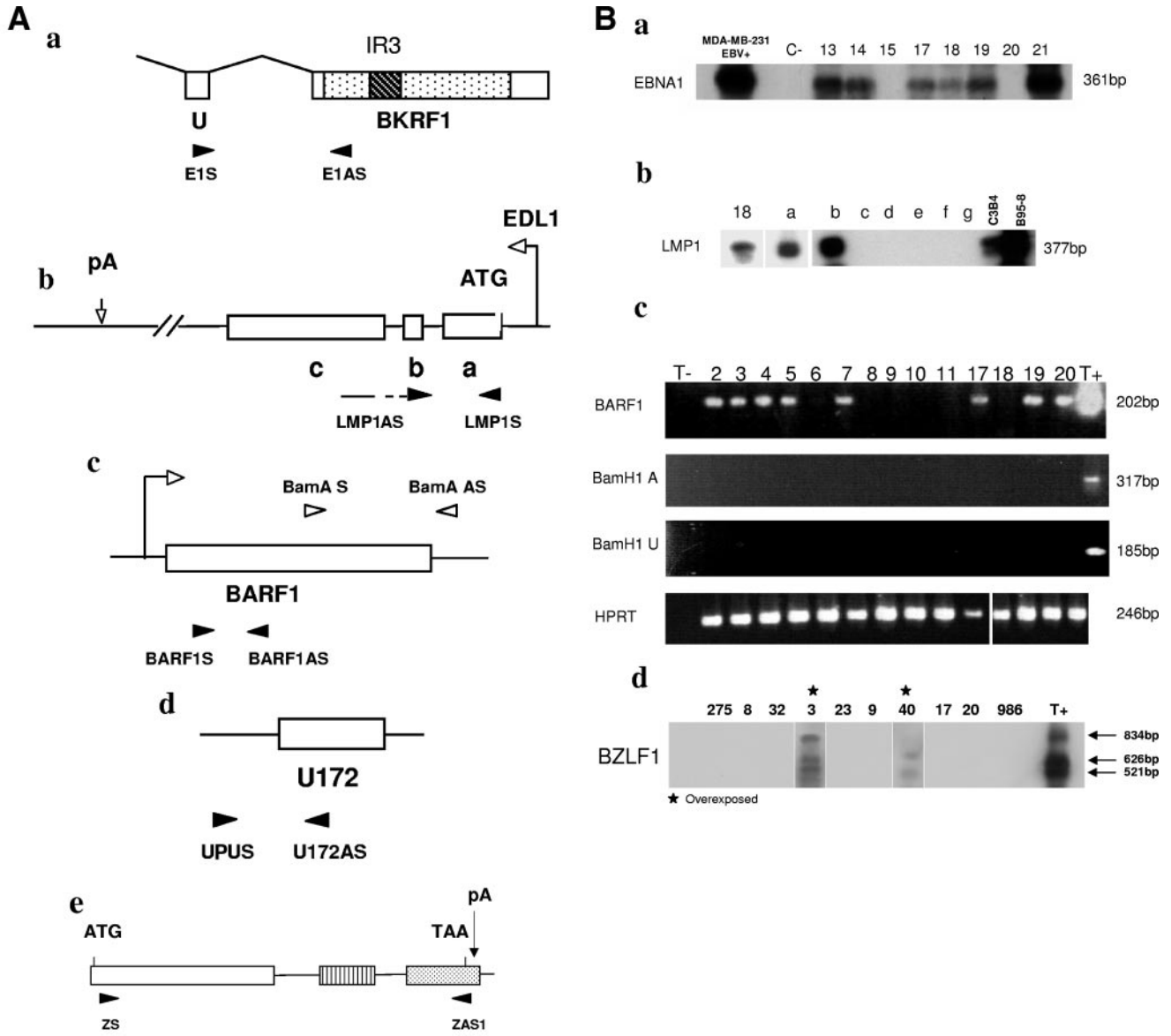

FIG. 2. Detection of EBV transcripts by RT-PCR analysis in RNA preparations from breast cancer biopsies. The primer combinations used are shown as arrows in the diagrams presented in panel A. For cDNA detection, the following primers were used: E1AS and E1S were used for EBNA-1 (a), LMP1S and LMP1AS for LMP-1 (b), and BARF1S BARF1AS for BARF-1 (c), and ZS and ZAS1 for BZLF1 (e). For detection of putative DNA contamination, BamAS and BamAAS (c) and U172AS and UPUS (d) were used as controls. Products obtained after RT-PCR are shown in panel B after Southern blotting (Ba, Bb, and Bd) or on agarose gels stained with ethidium bromide (Bc). Amplification of EBNA-1 cDNA is shown in panel Ba. MDA-MB-231 cells were used as the negative control, and EBV-infected MDA-MB-231 cells were used as the positive controls for EBNA-1 cDNA amplification. LMP-1 cDNA amplification is shown in panel Bb. B95-8 and C3B4 (an EBV-infected MDA-MB-231 cell line) served as positive controls. BARF-1 cDNA amplification is shown in panel Bc, and BZLF1 amplification is shown in panel Bd. B95-8 cDNA served as a positive control for BARF-1 and BZLF1, and DNA from B85-8 served as a positive control for DNA amplification in BamH1A and BamH1U.

contained 6,333 copies of EBV DNA in 1,000 total cells. As suggested by Huang et al. (15), lytic viral replication might contribute to the detection of the EBV genome in breast cancer, since Zta protein has been immunostained in a small number of cells from breast tumor. We detected BZLF1 transcripts in two of the eight breast cancer biopsy specimens. Moreover, proteins expressed during the productive cycle might be of importance for cell transformation: MMP9 is induced by BZLF1 (43), BRL1 induces expression of FAS (22) and activates S-phase entry through E2F1 induction (33), and BZLF1 interacts with cell cycle progression and is able to form complexes with p53 (45) and p65 (13), which play roles in the control of apoptosis. Moreover, lytic gene expression protects from cell death (17). However, it is also known that EBV reactivation occurs in a few cells in NPC (7) in which infection is predominantly latent, and such a phenomenon does not preclude latent infection.

Since identification of infected cells within a tumor has pre-

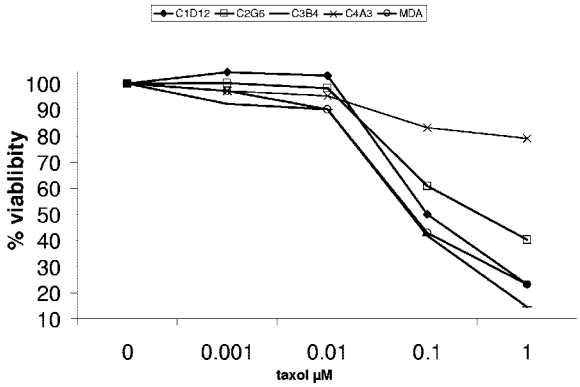

FIG. 3. Comparative toxicity of paclitaxel to MDA-MB-231 cells and to the different EBV-infected clones. Thirty thousand cells were seeded in 100 μl of culture medium in microtiter plates and incubated for 72 h with various concentrations of paclitaxel. The viability was assessed by means of the endogenous enzyme hexosaminidase. The experiment was performed in triplicate.

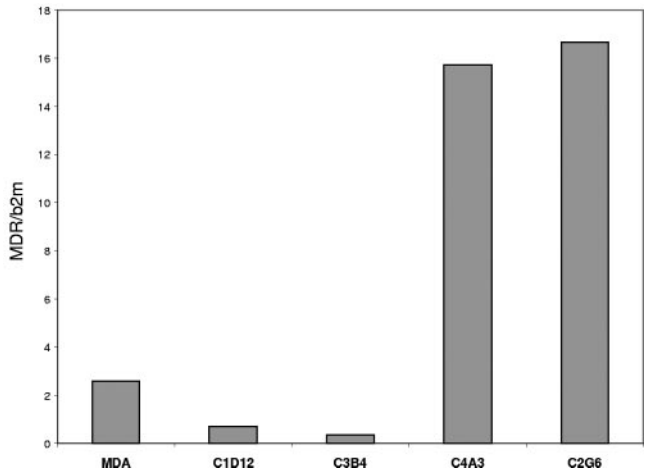

FIG. 4. EBV infection up-regulates MDR1 expression in two different EBV-infected MDA-MB-231 cell clones. Quantitative real-time RT-PCR analysis was performed on RNA extracted from cells exposed during 72 h to 0.1 μM paclitaxel; the transcript copy numbers relative to the reference gene β2-microglobulin (b2m) were determined.

viously given conflicting results in the literature, evidence of an association of EBV with breast cancer requires clarification. Negative results could arise from several sources: lack of assay sensitivity, storage of samples, use of fixed versus frozen tissues, and heterogeneity of the cancer tissue. False-positive results could arise from nonmonospecific antibody reagents or from EBV-positive infiltrating B lymphocytes, the level of which has been estimated to be 2 to 60 EBV-positive lymphocytes in 10^6 peripheral blood lymphocytes (25).

In order to test whether the detected EBV genomes were harbored only by lymphocytes, we investigated the viral loads in five pellets of peripheral blood lymphocytes from healthy blood donors (Table 2). As these results were negative, we concluded that most if not all of the EBV DNA detected in the breast cancer biopsy specimens was of epithelial tumor cell origin. We confirmed this conclusion by LCM analysis. Q-PCR performed on epithelial tumor cells purified from EBV-positive tumors by microdissection shows that the isolated tumor cells carry the viral genome and in much higher copy number in several of the microdissected specimens. Six to seven microdissections were also performed in the two biopsied specimens of breast cancer tissue by Fina et al. (10). Their results also showed heterogeneity of the viral load in samples containing only epithelial cancer cells and that infiltrating lymphocytes could not be the only source of the detected EBV.

In contrast, Murray et al. (27) failed to detect EBV genomes in microdissected tissue. However, they did not sample different areas of the same tumor but pooled all of the microdissected cells from each specimen. Thus, dilution of EBV-positive cells by noninfected cells may have resulted. Moreover, they analyzed fixed tissue, which resulted in lower sensitivity.

We show here quantification of EBV DNA in individual microdissections of four different tumors. A large heterogeneity in distribution of viral genomes from one region to another of the same tumor as well as among different tumors was observed. Moreover, the numbers of EBV genomes in some of the microdissected samples are not compatible with contamination with B lymphocytes, since only 1 to 10 per 10^6 are EBV positive. We also show that even when EBV is detected at a "high" level within one given region, the whole tumor may exhibit a very low viral load overall.

We have detected EBNA-1 staining with two EBNA-1 monoclonal antibodies (1H4, used for frozen sections, and 2B4, used on paraffin sections) of breast cancer tumors (3), and we also detected EBNA-1 transcripts in RNA taken from the same tumor. Xue et al. (41) detected EBV transcripts in samples sorted for the absence of lymphocytes. They detected EBNA-1 and BARF-1 RNA in three of six cases. We show here detection of EBNA-1, BARF-1, and LMP-1, respectively, in 17 of 22, 8 of 14, and 3 of 15 cases. Expression of EBNA-1, BARF-1, and LMP-1 genes is of importance. The EBNA-1 protein is required to replicate and maintain the EBV genome in infected cells, LMP-1 is considered to be the important EBV oncogene (18), and BARF-1 is able to confer transforming properties in cells (29). Both LMP-1 and BARF-1 are expressed in other carcinomas (32), in NPC and in gastric cancers.

In conclusion, it is well known that morphologically indistinguishable breast cancer cells in fact may comprise a heterogeneous population of breast cancer cells Similarly, we have now demonstrated that the distribution of EBV genomes in seemingly identical cells is heterogeneous. Because EBV is detected in only some breast cancer cells, it is unlikely to be a primary etiologic agent.

Herrmann and Niedobitek (14) questioned the implication of EBV in breast cancer, since the virus is detected in only a subset of tumor cells. However, EBV might have a role at an early step in carcinogenesis and then be lost after the development of the tumor. Alternatively, infection with EBV at a late state of tumor development might enhance oncogenic properties, such as invasiveness, angiogenesis, and metastasis. For example, it has been shown that LMP-1 induces matrix metalloproteinase 9 (MMP9), cyclooxygenase-2 (COX2), and vascular endothelial growth factor (VEGF) (26) and induces and causes release of FGF-2 in human epithelial cells (39). Moreover, a correlation between LMP-1 expression and the presence of COX2 in NPC tissue was observed. These findings

raise the possibility that EBV might alter the phenotype of a subpopulation of carcinomatous cells so that they become more aggressive in behavior (40). Our results show that EBV infection confers paclitaxel resistance to breast carcinoma cell lines. This resistance might be a consequence of the higher MDR1 expression that is observed in paclitaxel-resistant EBV-infected cells (C4A3 and C2G6). Moreover, MDR is induced by paclitaxel treatment in the C4A3 cells. The manner by which the cells acquired high MDR1 gene expression has not yet been defined. Overexpression of the MDR1 gene product may be intrinsically expressed or acquired following chemotherapeutic drug treatment. Different molecular mechanisms may lead to MDR1 overproduction: increased mRNA stability, increased translational initiation (42), hypomethylation of the promoter (34), and changes in the spatial and temporal pattern of histone modifications (1). How EBV affects those mechanisms is yet to be uncovered. Overexpression of the MRD1 gene upon EBV infection might have an effect on therapeutic treatment of the disease.

Whether EBV might have a role in breast cancer development or progression now needs to be promptly investigated with appropriate cell biologic tools.

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