Title

Bovine leukemia virus linked to breast cancer but not coinfection with human papillomavirus: Case-control study of women in Texas

Journal

Cancer, 124(7)

ISSN

0008-543X

Authors

Baltzell, Kimberly A
Shen, Hua Min
Krishnamurthy, Savitri
et al.

Publication Date

2018-04-01

DOI

10.1002/cncr.31169

Peer reviewed

Original Article

Bovine Leukemia Virus Linked to Breast Cancer But Not Coinfection With Human Papillomavirus: Case-Control Study of Women in Texas

Kimberly A. Baltzell, RN, PhD, MS [1]; Hua Min Shen, MD[2]; Savitri Krishnamurthy, MD[3]; Jennette D. Sison, MPH[4]; Gerard J. Nuovo, MD[5]; and Gertrude C. Buehring, PhD[2]

BACKGROUND: Bovine leukemia virus (BLV) and human papillomavirus (HPV) were previously identified in human breast tissue and have been associated with breast cancer in independent studies. The objective of the current study was to test for the presence of BLV and HPV in the same breast tissue specimens to determine whether the viruses were associated with breast cancer either singly or together. **METHODS:** Archival formalin-fixed paraffin-embedded breast tissue sections from 216 women were received from The University of Texas MD Anderson Cancer Center along with patient diagnosis. In situ polymerase chain reaction and/or DNA hybridization methods were used to detect targeted DNA segments of BLV and HPV. Standard statistical methods were used to calculate age-adjusted odds ratios, attributable risk, and *P* values for the trend related to the association between presence of a virus and a diagnosis of breast disease. **RESULTS:** Women diagnosed with breast cancer were significantly more likely to have BLV DNA in their breast tissue compared with women with benign diagnoses and no history of breast cancer. Women with breast pathology classified as premalignant and no history of breast cancer also were found to have an elevated risk of harboring BLV DNA in their breast tissue. HPV status was not associated with malignancy, premalignant breast disease, or the presence of BLV in the breast tissues. **CONCLUSIONS:** The data from the current study supported previous findings of a significant association between BLV DNA in breast tissue and a diagnosis of breast cancer, but did not demonstrate oncogenic strains of HPV associated with breast cancer or the presence of BLV DNA in breast tissue. The authors believe the findings of the current study contribute to overall knowledge regarding a possible causal role for viruses in human breast cancer. ***Cancer*** **2018;124:1342-9.** *© 2017 American Cancer Society.*

KEYWORDS: breast cancer, bovine leukemia virus (BLV), case-control study, human papillomavirus (HPV), oncogenic viruses.

INTRODUCTION

Can breast cancer be caused by a virus? This question has permeated the minds of researchers for the last 40 to 50 years since the discovery that mammary cancer in mice was caused by a milk-borne virus transmitted from mother to pups. Breast cancer clearly is a multifaceted disease with numerous risk factors contributing to the final outcome. However, identifying the strong involvement of a virus would enable several prevention options. Although the prevention of most cancers is a secondary prevention (ie, early detection and treatment of the developing disease), the enormous medical and humanitarian payoffs of primary prevention (ie, prevention of the initiation of the disease) emphasize the need to encourage and support research concerning earlier prevention strategies. Cancers caused by infectious agents are particularly fortuitous in that the successful primary prevention of many noncancerous infectious diseases has provided useful precedents for primary prevention. Stellar examples are the vaccine prevention of hepatocellular carcinoma caused by hepatitis B virus (HBV), and the more recently developed vaccines against certain strains of human papillomavirus (HPV), which causes cancer of the uterine cervix, anal cancer, and head and neck cancers.[1] The success of the antimicrobial treatment of *Helicobacter pylori* infection to prevent ulcers and subsequent stomach cancer is another victory for primary prevention through a relatively straightforward solution.[1]

The viruses most extensively explored as possible players in human breast cancer are mouse mammary tumor virus and mouse mammary tumor virus-like sequences, Epstein-Barr virus, HPV, and bovine leukemia virus (BLV). Despite numerous publications,[2,3] to the best of our knowledge there is as yet no consensus regarding a viral role in either the

Corresponding author: Kimberly A. Baltzell, RN, PhD, MS, Department of Family Health Care Nursing, University of California at San Francisco, 2 Koret Way, N431Y, San Francisco, CA 94143; kimberly.baltzell@ucsf.edu

[1]Department of Family Health Care Nursing, University of California at San Francisco, San Francisco, California; [2]School of Public Health, University of California at Berkeley, Berkeley, California; [3]Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas; [4]Department of Neurological Surgery, School of Medicine, University of California at San Francisco, San Francisco, California; [5]Department of Pathology, Ohio State University Comprehensive Cancer Center, Columbus, Ohio

We thank Anita L. Vines of The University of Texas MD Anderson Cancer Center for organization and shipping of the specimens, Steve Ruzin and Diane Tran of the University of California at Berkeley for assistance with formatting the figures, and Kan Tong of the University of California at Berkeley for reference checking.

DOI: 10.1002/cncr.31169, **Received:** June 28, 2017; **Revised:** September 11, 2017; **Accepted:** October 24, 2017, **Published online** December 20, 2017 in Wiley Online Library (wileyonlinelibrary.com)

initiation and/or maintenance of human breast cancer. Our focus in the current study was to examine human breast tissues for the presence of BLV, a deltaretrovirus that to our knowledge is less studied, but its presence in human breast tissue[4] and significant association with breast cancer[5] warrant further investigation. The prevalence of BLV in US cattle is 47.6% of dairy cows and 33.6% of beef cattle.[6] Because close to 84% of all large dairy herds in the United States and 78% of dairy herds in Canada have some BLV-positive cattle within the herd,[7] and because milk from all cows usually is pooled before it goes to market, it is difficult to avoid exposure to BLV if milk is part of one's diet. Although pasteurization inactivates BLV, the consumption of raw milk dairy products and undercooked beef is not uncommon and transmission from already infected humans also is plausible. BLV is readily transmitted through the herd via milk and blood routes. In humans, epidemiologists have long noted that countries with the highest milk consumption also had the highest breast cancer incidence.[5]

In addition to BLV, we also tested for HPV in adjacent sections of the same breast specimens representing different pathology classifications of breast tissue. Because HPV frequently has been identified as coinfecting the same tissues with other viruses and possibly having a synergistic effect,[8] we examined the relative frequencies of BLV and HPV to determine whether these viruses, either singly or together, are associated with a history of breast cancer.

MATERIALS AND METHODS

Breast tissue specimens were acquired from the archives of The University of Texas MD Anderson Cancer Center (MDACC). Before surgery or biopsy, patients provided informed consent for the disposition of unused tissue to be at the discretion of the pathology laboratory. The human subjects protocol was approved by the institutional review boards of MDACC, the University of California at San Francisco, and the University of California at Berkeley. Patients eligible for the study were those aged $\geq$18 years who were undergoing a breast procedure (biopsy or excision). Specimens were accessioned from 2009 through 2011 from 218 women, were processed within 24 hours after surgery to minimize any DNA degradation, and were fixed in 10% buffered neutral formalin and embedded in paraffin. Tissue sections (5-μm thick) were cut at MDACC; sections stained with hematoxylin and eosin were retained for routine pathology examination, 4 unstained sections were shipped to the University of California at Berkeley for in situ polymerase chain reaction (IS-PCR) assays for BLV and HPV type 16 (HPV-16), and 2 sections were shipped to the Ohio State University Comprehensive Cancer Center for the in situ hybridization (ISH) assay (Ventana Medical Systems, Tucson, Arizona) for HPV DNA (12 oncogenic strains). Two subjects were eliminated from the study due to receipt of broken slides, small size of the tissue specimen, and/or a lack of mammary epithelial cells in the specimen, leaving a final sample of 216 specimens. All specimens were selected for the current study and the pathology classification determined by the team pathologist (S.K.) as part of the routine clinical diagnosis of the patients. The tumors were graded using the combined Nottingham histologic grading system.[9] Cells were classified as premalignant according to the guidelines of the Cancer Committee of the College of American Pathologists based on the relative risk of developing breast cancer for women diagnosed with particular nonmalignant breast changes. The premalignant category consisted of tissue diagnosed as ductal or lobular carcinoma in situ, and atypical hyperplasia of ductal or lobular origin. Fibrocystic change, flat epithelial atypia, and normal hyperplasia are not considered premalignant and therefore were included in the benign (normal) category, which served as the control against which the premalignant and malignant categories were compared.

Direct IS-PCR, as adapted from Nuovo,[10] was used to detect the presence of retrotranscribed BLV DNA directly in formalin-fixed breast tissue sections and to identify which cell types were BLV positive. This technique was used previously to detect BLV in bovine,[11] alpaca,[12] and human tissues,[4,5] and differs from standard-solution PCR and real-time (RT) PCR (RT-PCR) in several ways: 1) no DNA is extracted; formalin-fixed sections are mounted on super-adherent glass slides, deparaffinized, and permeabilized before the reaction is performed; 2) the PCR mix has a different composition from that used in the standard-solution and RT-PCR methods; 3) cycling times are longer because glass slides are thicker than the tubes used for standard-solution PCR and RT-PCR; and 4) the thermal cycler used must be designed specifically to accommodate slides rather than tubes. IS-PCR was performed as described previously in detail.[4] The positive control cell line for BLV was the FLK (fetal lamb kidney) cell line,[13] authenticated as sheep using sheep-specific primers based on the cytochrome C oxidase gene.[14] The positive control cell line for HPV-16 was CaSki,[15] authenticated as human by the presence of the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) enzyme. Thick suspensions of detached, rinsed

cells from control cell lines were smeared on enhanced adherence glass microscope slides, air dried, and fixed for 16 to 18 hours with 10% buffered neutral formalin. To enhance entry of the PCR mix into formalin-fixed cells, samples were permeabilized by digestion with 2 mg/mL of pepsin in 0.1 N HCl (60 minutes for tissue sections and 20 minutes for control cell smears) and pepsin inactivation solution (100 mmol/L of Tris-HCl and 100 mmol/L of NaCl [pH 7.4]) applied for 1 minute, followed by a Dulbecco modified phosphate-buffered saline rinse and 5 minutes in absolute ethanol. Samples were surrounded with a 15 mm × 15 mm frame seal chamber (Bio-Rad, Hercules, California) and 60 μL of PCR mix then was placed into the chamber. After the addition of the PCR mix, the plastic cover was sealed by pressing tightly over the frame. Slides were placed into an IS-PCR machine (Hybaid OmniSlide; Cambridge Biosystems, Cambridge, United Kingdom) for amplification.

The specific primer sequences for BLV, shown below, were from the *tax* region of BLV, amplifying a 114-base pair (bp) product. Their genomic location in bp numbering is according to GenBank accession number EF600696: forward (bp 7310-7329): ATGTCACCATCGATGCCTGG; and reverse (bp 7423-7404): CATCGGCGGTCCAGTTGATA.

The IS-PCR mix for BLV was 4.0 mmol/L of $MgCl_2$; 0.4 mmol/L of dNTPs; 1 μmol/L of primers (Operon Biotechnologies, Huntsville, Alabama); 0.06% bovine serum albumin, 8 μmol/L of digoxigenin-11-dUTP (Hoffman-La Roche, Basel, Switzerland); and 0.053 U/μL of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, California), a Taq polymerase activated only at ≥92 °C, designed to reduce false-positive results from nonspecific DNA repair by Taq at cooler temperatures.

Primer sequences for HPV-16 were from the L1 capsid region and amplified a 105-bp product: forward (bp 1288-1311): TTTGGTCTACAACCTCCCCCAGGA) and reverse (bp 1392-1369): TTCTTTAGGTGCTGGA GGTG TATG).

The IS-PCR reaction mix for HPV-16 was 4.5 mM of $MgCl_2$, 0.4 mM of deoxyribonucleoside triphosphates, 1 μM of primers (synthesized by Operon Biotechnologies), 8 μM of digoxigenin-11-deoxyuridine triphosphate, 0.06% bovine serum albumin, and 0.05 U/μL of Taq polymerase.

Cycling parameters for both viruses were 1 cycle at 93 °C for 10 minutes, at 92 °C for 2 minutes, and at 57 °C for 1.5 minutes; for 30 cycles at 92 °C for 30 seconds, at 57 °C for 1.5 minutes, and at 69 °C for 2 minutes; and for 1 cycle at 69 °C for 10 minutes. On completion of amplification, covers and chambers were removed and slides were rinsed in Dulbecco modified phosphate-buffered saline and endogenous peroxidase was quenched for 30 minutes in 3% hydrogen peroxide in methanol. Label incorporated into PCR products was detected by antidigoxigenin-11-dUTP antibodies in an avidin-biotin-immunoperoxidase reaction (Hoffman-La Roche). The chromogen was diaminobenzidine (Sigma Chemical Company, St. Louis, Missouri). The outcome measurement was a semiquantitative judgement of the brown color density of cells: light tan indicated 1+, medium tan indicated 2+, dark brown indicated 3+, and almost black indicated 4+. Ratings of ≥ 2 + were considered as positive. Slides were independently read by 2 individuals (H.M.S. and G.C.B.).

Initially, the BLV-positive control cell line (FLK) and the BLV-negative cell line (Tb_1Lu) were used to optimize the reaction and ensure no false-positive reaction in the negative cell line.[4] Controls run simultaneously with each batch of human tissue assays were: 1) a positive control (a smear of BLV-positive cells [FLK cell line] reacted with complete PCR mix); and 2) background controls (a smear of FLK cells and an adjacent serial section of each specimen reacted with PCR mix minus primers) to rule out potential false-positive reactions, unique to each tissue, resulting from unquenched endogenous peroxidase, nonspecific reaction of the sheep antibodies used in the final immunoperoxidase detection, nonspecific DNA repair by Taq polymerase, and/or the presence of brown pigmented cells (melanocytes, hair follicles) occasionally found in breast tissues.

HPV DNA ISH was performed using a previously published protocol.[16] In brief, the Ventana ISH iVIEWBlue Plus Detection Kit was used (Ventana Medical Systems). After pretreatment with protease digestion, the tissue DNA was denatured simultaneously with the probes for high-risk HPV types and hybridized overnight, and the signal then visualized with an alkaline phosphatase conjugate on NBT/BCIP (nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolephospate p-toluidine) with a nuclear fast counterstain. Positive controls included cervical intraepithelial neoplasia lesions with known HPV types, as determined by DNA sequencing. Negative controls were histologically normal uterine cervical tissues.

Statistical Analysis

The data analysis output for this investigation was generated using SAS statistical software (version 9.4; SAS Institute Inc, Cary, North Carolina). PROC FREQ (SAS

TABLE 1. Population Demographics

		Diagnostic Category				
Population Categories ↓	All Women	Benign (Controls)	Malignant (Cases)	Malignant Plus DCIS	Premalignant	Malignant Plus Premalignant
No. (%)	216	102 (47.22)	61 (28.24)	90 (41.67)	53 (24.54)	114 (52.78)
Age at time of diagnosis, y						
No. of subjects with age information available	210	96	61	90	53	114
Age range, y	30-81	30-78	32-77	32-77	37-81	32-81
Mean	54.63	54.4	54.59	55.22	55.11	54.89
Median ± SE	53.5 ± 0.718	53.0 ± 1.040	53.0 ± 1.440	54.0 ± 1.153	54.0 ± 1.366	54.0 ± 1.020
PROC T test *P* for cases vs controls			.9111	.5943	.6788	.7622
Ethnicity/race, no. (% of total)						
White	147 (68.06)	71 (69.61)	41 (67.21)	59 (65.56)	35 (66.04)	76 (66.76)
African American	19 (8.80)	9 (8.82)	4 (6.56)	7 (7.78)	6 (11.32)	10 (8.77)
Arabic	2 (0.93)	2 (1.96)	0	0	0	0
Asian	14 (6.48)	6 (5.88)	4 (6.56)	7 (7.78)	4 (7.55)	8 (7.02)
Hispanic	24 (11.11)	6 (5.88)	11 (18.03)	16 (17.78)	7 (13.21)	18 (15.79)
Unknown or missing data	10 (4.63)	8 (7.84)	1 (1.64)	1 (1.11)	1 (1.89)	2 (1.75)
P	.4292[a]		.1677[b]	.0888[b]	.5182[b]	.1295[b]
Whites only	147 (68.06)	71 (69.61)	41 (67.21)	59 (65.56)	35 (66.04)	76 (66.76)
All other races	59 (27.31)	23 (22.54)	19 (31.15)	30 (33.33)	17 (32.08)	36 (31.58)
Unknown or missing data	10 (4.63)	8 (7.84)	1 (1.64)	1 (1.11)	1 (1.89)	2 (1.75)
P	.4754[a]		.3280[a]/.3572[b]	.1684[a]/.1937[b]	.2860[a]/.3341[b]	.2249[a]/.2790[b]

Abbreviations: DCIS, ductal carcinoma in situ; SE, standard error.
[a] *P* value was derived using the chi-square test.
[b] *P* value was derived using the Fisher exact test.

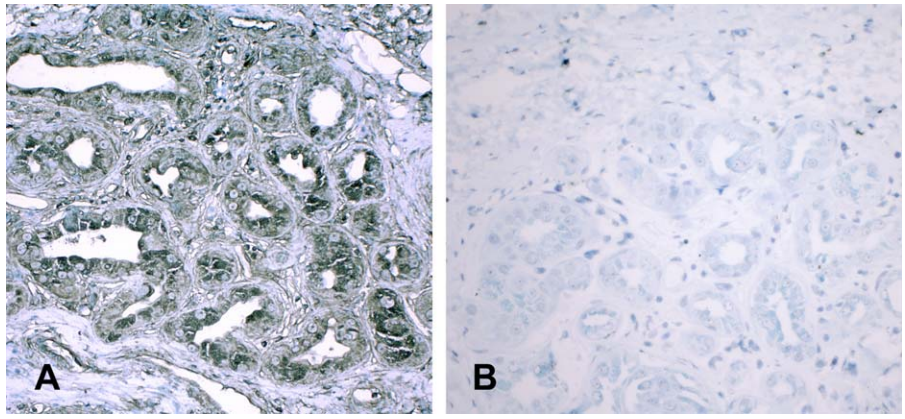

Figure 1. Bovine leukemia virus (BLV)-positive mammary epithelial cells. Breast tissue diagnosed as benign demonstrated cross-sections of normal lobules. (A) An intense BLV *tax* DNA segment signal (dark brown-black) was noted in the cytoplasm of many epithelial cells, especially the luminal portion. No definitive nuclear reaction was apparent. (B) An adjacent section of the same tissue reacted with polymerase chain reaction mix minus primers as a background control to reveal potential artifacts that might resemble a positive reaction (eg, melanin, excess peroxidase, nonspecific Taq polymerase DNA repair activity). No dark brown signals were apparent. Both sections were lightly counterstained with Diff-Quik blue (original magnification × 40).

Institute Inc) was used for frequency distributions, as well as chi-square and Fisher exact tests to compare groups for significant differences among categorical variables. The PROC T test (SAS Institute Inc) was used to ascertain the significant difference among the groups with respect to age. Odds ratios (ORs) were obtained using PROC LOGISTIC (SAS Institute Inc) and 95% confidence intervals (95% CIs) were calculated for ORs. The PROC FREQ Cochran-Armitage test for trend option (SAS Institute Inc) was used to detect an increasing trend of viral presence from benign to malignant conditions among specimens. All final analyses were adjusted for age and ethnicity when appropriate. A significance level of 95% was set at an $\alpha \leq .05$.

TABLE 2. Risk Comparisons Between Diagnostic Groups and Controls

BLV Status	Total Population N=214	Controls (Benign) N=103	Cases (Malignant) N=61	Cases Plus DCIS N=89	Cases Plus All Premalignancies N=112	Premalignant N=52
			Diagnostic Category No. of Women (% of Total)			
Positive (% positive)	73 (33.80)	20 (19.61)	35 (57.38)	49 (54.44)	53 (46.49)	18 (33.96)
Negative (% negative)	141 (64.28)	83 (78.43)	26 (42.62)	41 (45.56)	61 (53.51)	32 (66.04)
Comparison with controls		*P* OR (95% CI)	<.0001[a]/.0001[b]	<.0001[a]/<.0001[b]	<.0001[a]/<.0001[b]	.0572[a]/.0763[b]
			5.87 (2.83-12.16)	5.25 (2.69-10.23)	3.80 (2.01-7.18)	2.24 (1.03-4.84)
HPV Status by IS-PCR						
Positive (% positive)	14 (6.48)	8 (7.84)	2 (3.28)	3 (3.33)	6 (5.26)	4 (7.55)
Negative (% negative)	200 (92.59)	92 (90.20)	59 (96.72)	87 (96.67)	108 (94.74)	49 (92.45)
Comparison with controls		*P*	.2286[a]/.3211[b]	.1691[a]/.2201[b]	.1492[a]/.5811[b]	.9210[a]/1.0000[b]
HPV Status By ISH						
Positive (% positive)	11 (5.09)	3 (2.94)	4 (6.56)	7 (7.78)	8 (7.02)	4 (7.55)
Negative (% negative)	189 (87.50)	92 (90.20)	56 (91.80)	78 (86.67)	97 (85.09)	41 (77.36)
Comparison with controls		*P*	.3055[a]/.4310[b]	.1376[a]/.1946[b]	.1670[a]/.2201[b]	.1462[a]/.2113[b]

Abbreviations: 95% CI, 95% confidence interval; BLV, bovine leukemia virus; DCIS, ductal carcinoma in situ; HPV, human papillomavirus; ISH, in situ hybridization; IS-PCR, in situ polymerase chain reaction; OR, odds ratio.

[a] *P* value was derived using the chi-square test and adjusted for age and race (white/nonwhite).

[b] *P* value was derived using the Fisher exact test.

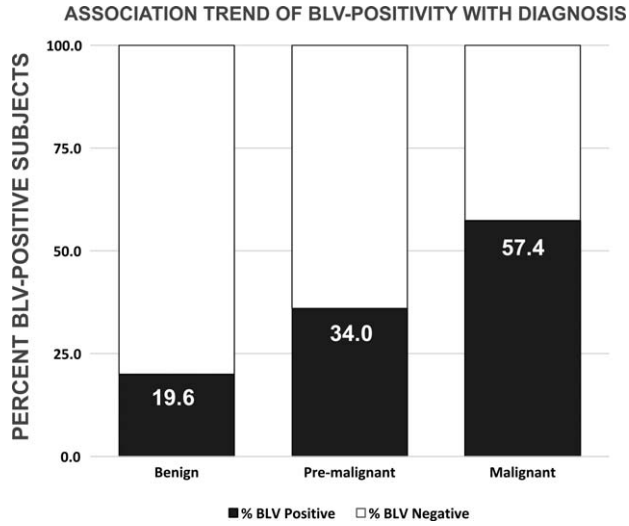

Figure 2. Association trend of bovine leukemia virus (BLV) positivity with diagnosis. Benign indicates no evidence of premalignant or malignant changes and no history of breast cancer (103 patients). Premalignant indicates premalignant changes but no malignant changes and no history of breast cancer (52 patients). Malignant indicates malignant changes in the tissue and a clinical diagnosis of breast cancer (61 patients). Note the stepwise increase in the frequency of detection of the BLV *tax* DNA segment with disease progression (*P* for trend, < .0001).

RESULTS

Table 1 summarizes the demographic characteristics of the study population. The 5 diagnostic categories of subjects, classified by their clinical diagnosis and the pathology of their tissue specimen, were remarkably well matched with regard to age, with no statistically significant differences noted. With regard to ethnicity/race, subjects classifying themselves as "white" comprised the majority (68%), with other races/ethnicities combined comprising 32% of the total.

Of the total population of 214 subjects in all diagnostic categories and with available demographic information, 73 (34%) were positive for the targeted BLV *tax* DNA segment. Figure 1 is an example of a positive reaction obtained by IS-PCR in normal breast lobules, which illustrate the positive reaction better than malignant tissues because normal tissue has a relatively uniform cell size and arrangement. Our main finding was that women diagnosed with breast cancer were significantly more likely to have the targeted BLV *tax* DNA segment in their breast tissue (age/ethnicity-adjusted OR, 5.87; 95% CI, 2.83 to 12.16) compared with women with benign diagnoses and no history of breast cancer. The population-attributable risk was 51.82%. Women with breast pathology classified as premalignant

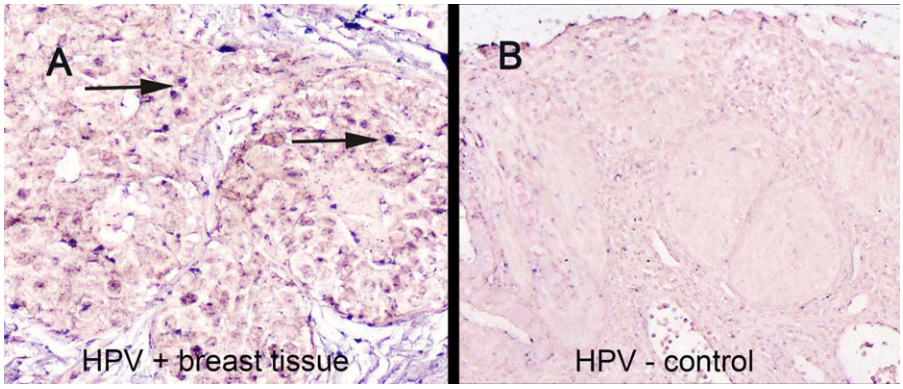

Figure 3. Human papillomavirus (HPV)-positive mammary epithelial cells. (A) Breast tissue diagnosed as benign lobular hyperplasia demonstrated cross-sections of lobules with multiple epithelial layers. The nuclei of many epithelial cells demonstrated a dark punctate pattern typical of an HPV-positive reaction (arrows) by in situ hybridization. (B) HPV-negative uterine cervix tissue with squamous metaplasia was used as a negative control for the reaction. No dark punctate pattern was apparent in the nuclei. Nuclear fast red was used as the counterstain for both specimens (original magnification × 40).

also had an elevated risk of harboring the targeted BLV segment in breast tissue (age-adjusted OR, 2.24; 95% CI, 1.03-4.84). The details are summarized in Table 2. Significant frequency differences in breast tissue BLV status also occurred between women in various diagnostic categories: breast cancer versus premalignant (chi-square test $P = .025$; Fisher exact test $P = .03$) and premalignant versus benign (chi-square test $P = .03$). Figure 2 illustrates the stepwise progression in the frequency of the BLV *tax* DNA segment going from the benign to premalignant to malignant categories (P for trend $< .0001$, 2-sided Cochran-Armitage trend test).

However, among malignant specimens only, BLV status was not found to be associated with increasing tumor grade.

HPV was found in only 14 of 214 specimens (7%). Figure 3 illustrates a positive HPV ISH reaction. HPV status was not found to be associated with malignancy or premalignancy. In fact, more benign specimens were HPV positive for the targeted HPV genome segment than those diagnosed as malignant or premalignant, but the differences were not statistically significant. There was no significant difference noted between the presence of HPV in BLV-positive versus BLV-negative tissues. Details are summarized in Table 2.

DISCUSSION

Five viruses are widely accepted by the scientific community as causative agents of human cancers: HBV and hepatitis C virus for hepatocellular carcinoma; HPV for cancers of the uterine cervix, penis, vulva, periungual area, and head/neck; Epstein-Barr virus for Burkitt lymphoma; human herpesvirus 8 for Kaposi sarcoma; and Merkel cell polyomavirus for Merkel cell carcinoma. For hepatocellular carcinoma resulting from HBV and cervical carcinoma, which are not primarily found in immunosuppressed individuals, vaccines already have been developed and are providing effective primary prevention. It stands to reason that additional cancers with unknown etiologies eventually might be shown to have a viral etiology, and investigations in this area are worth pursuing.

There are several strengths to the current study. First, the primers used for IS-PCR are the most likely to detect the presence of BLV. Upon entry into a cell, deltaretroviruses use their polymerase (reverse transcriptase) to transcribe a DNA copy of their RNA genome. As the diseases they cause progress, genome regions coding for the polymerase, capsid, and envelope proteins of the viruses become increasingly deleted,[17,18] presumably to escape the host's immune response. For BLV in cattle and human T-cell leukemia virus, a deltaretrovirus closely related to BLV, all that may be left of the viral genome in advanced phases of the diseases are the long terminal repeat promoter region and the *tax* region coding for the oncogenic protein. The long terminal repeat promoter region demonstrates far greater sequence variation among individuals than *tax*, the most highly conserved of the BLV genome regions.[19] This infrequency in sequence variation leads us to believe that the same *tax* primers would detect this region in the majority of specimens. Support for the specificity of these primers for BLV is 2-fold. First, National Center for Biotechnology Information GenBank BLAST searches comparing the primer sequences with all viral

sequences in the nucleotide database[20] indicated high homology only with BLV (E = 0.23) and extremely low homology (E = 16.0 for the forward primer and 255.0 for the reverse primer) with human genome sequences, which include endogenous retroviruses. The E value is a measure of chance similarity; values < 1.00 have high homology, whereas values >1.00 have low homology with the sequence being tested. Second, a previous study demonstrated that these same *tax* primers failed to amplify representatives of all oncogenic retroviral families, lentiviruses, and viruses reported to be present in human breast tissues (HPV, Epstein-Barr virus, and human endogenous retrovirus K [HERV-K]).[4] The genome region of HPV chosen as a target for PCR was from the capsid region and has been the most commonly used target for previous studies regarding cancer of the uterine cervix and breast cancer. Due to the need to preserve archival tissue in the pathology department that provided the specimens, it was not possible to obtain further sections with which to examine additional genome areas of the 2 viruses.

Another strength of the current study is the IS technique. Cross-contamination with the positive control cell line or among specimens was impossible because the DNA was not extracted and was fixed within the control cells and human tissues and could not escape to cross-contaminate. A further advantage is that IS-PCR allows for localization of the PCR amplification to particular cell types.

A third strength of the current study is the epidemiology reported herein. The cases, controls, and premalignant subjects were extraordinarily well matched with regard to age (Table 1), which is difficult to do in many investigations involving human subjects. Thus, despite a moderate sample size, the results appear to be clear cut, with high ORs, and the lower boundaries of the 95% CIs were > 1.00 (Table 2).

Although many steps are necessary to establish causation of any disease, a statistically significant association between the disease and the suspected agent is the most essential first step, and validation of this association by other investigators in different populations is an essential subsequent step.[21] The data presented in the current study indicate a highly significant relationship between the presence of the targeted BLV *tax* DNA segment and human breast cancer in women residing in Texas, thus corroborating the results of a previous study regarding a different population of US women.[5] The attributable risk of 51.82% indicates that BLV could be responsible for at least one-half of the breast cancer cases in the population studied. The significant *P* value for trend (<.0001) is consistent with the well-accepted idea that breast cancer is a gradual process of cellular transition from normal to cancer, and it adds a new perspective that persistent oncogenic viral infection could play a role in tumor progression. Previous evidence that the oncogenic protein Tax in BLV can inhibit DNA repair[22] suggests one mechanism by which BLV could initiate breast cancer over a period of years as individual BLV-infected cells accumulate unrepaired genomic mutations.

However, the data from the current study do not support oncogenic strains of HPV playing an etiologic role in breast cancer, even though 2 methods of HPV detection were used. One method amplified a targeted DNA segment of the most common HPV type involved in cervical cancer (HPV-16) using IS-PCR. The other method used a commercial kit (Ventana Medical Systems) that frequently is used in the clinical diagnosis of uterine cervical infections that targeted 12 oncogenic PCR types using ISH without PCR amplification. The low frequency of HPV detection (<7%) noted with both methods in all diagnostic categories does not support the belief that HPV plays a significant role in breast cancer, either alone or synergistically with BLV. This is consistent with the findings of several previous studies regarding HPV and breast cancer.[2,23]

FUNDING SUPPORT

Supported by a grant from the Avon Foundation for Women (grant 02-2011-103).

CONFLICT OF INTEREST DISCLOSURES

Kimberly A. Baltzell reports grants from the Avon Foundation for work performed as part of the current study.

AUTHOR CONTRIBUTIONS

Kimberly A. Baltzell and **Gertrude C. Buehring** were responsible for the development and overall conduct of the study, as well as the writing of the article. **Hua Min Shen, Savitri Krishnamurthy, Gerard J. Nuovo**, and **Gertrude C. Buehring** were responsible for conducting all experiments. **Kimberly A. Baltzell** was responsible for data analysis and interpretation along with **Jennette D. Sison**.

REFERENCES

1. Vineis P, Wild CP. Global cancer patterns: causes and prevention. *Lancet*. 2014;383:549-557.
2. Joshi D, Buehring GC. Are viruses associated with human breast cancer? Scrutinizing the molecular evidence. *Breast Cancer Res Treat*. 2012;135:1-15.
3. Salmons B, Gunzburg WH. Revisiting a role for a mammary tumor retrovirus in human breast cancer. *Int J Cancer*. 2013;133:1530-1535.
4. Buehring GC, Shen HM, Jensen HM, Choi KY, Sun D, Nuovo G. Bovine leukemia virus DNA in human breast tissue. *Emerg Infect Dis*. 2014;20:772-782.

5. Buehring GC, Shen HM, Jensen HM, Jin DL, Hudes M, Block G. Exposure to bovine leukemia virus is associated with breast cancer: a case-control study. *PLoS One*. 2015;10:e0134304.
6. Bauermann FV, Ridpath JF, Dargatz DA. Bovine leukemia virus seroprevalence among cattle presented for slaughter in the United States. *J Vet Diagn Invest*. 2017;29:704-706.
7. Gyles C. Should we be more concerned about bovine leukemia virus? *Can Vet J*. 2016;57:115-116.
8. Guidry JT, Scott RS. The interaction between human papillomavirus and other viruses. *Virus Res*. 2017;231:139-147.
9. Pinder SE, Murray S, Ellis IO, et al. The importance of the histologic grade of invasive breast carcinoma and response to chemotherapy. *Cancer*. 1998;83:1529-1539.
10. Nuovo GJ. PCR in situ hybridization. In: Spector S, Bendinelli M, Friedman H, eds. Rapid Detection of Infectious Agents. New York: Plenum Press; 1998.
11. Duncan RB Jr, Scarratt WK, Buehring GC. Detection of bovine leukemia virus by in situ polymerase chain reaction in tissues from a case of sporadic bovine lymphosarcoma. *J Vet Diagn Invest*. 2005;17:190-194.
12. Lee L, Scarratt WK, Buehring GC, Saunders GK. Bovine leukemia virus infection in a juvenile alpaca with multicentric lymphoma. *Can Vet J*. 2012;53:283-286.
13. Van Der Maaten MJ, Miller JM. Replication of bovine leukemia virus in monolayer cell cultures. *Bibl Haematol*. 1975;43:360-362.
14. Cooper JK, Sykes G, King S, et al. Species identification in cell culture: a two-pronged molecular approach. *In Vitro Cell Dev Biol Anim*. 2007;43:344-351.
15. Pattillo RA, Hussa RO, Story MT, Ruckert AC, Shalaby MR, Mattingly RF. Tumor antigen and human chorionic gonadotropin in CaSki cells: a new epidermoid cervical cancer cell line. *Science*. 1977;196:1456-1458.
16. Nuovo G, ed. In Situ Molecular Pathology and Co-Expression Analyses. 1st ed. New York: Elsevier; 2013.
17. Gillet N, Florins A, Boxus M, et al. Mechanisms of leukemogenesis induced by bovine leukemia virus: prospects for novel anti-retroviral therapies in human. *Retrovirology*. 2007;4:18.
18. Kamihira S, Sugahara K, Tsuruda K, et al. Proviral status of HTLV-1 integrated into the host genomic DNA of adult T-cell leukemia cells. *Clin Lab Haematol*. 2005;27:235-241.
19. McGirr KM, Buehring GC. Tax & rex: overlapping genes of the Deltaretrovirus group. *Virus Genes*. 2006;32:229-239.
20. National Center for Biotechnology Information, US National Library of Medicine, National Institutes of Health. Standard nucleotide BLAST. https://blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed November 15, 2017.
21. Hill AB. The environment and disease: association or causation? *Proc R Soc Med*. 1965;58:295-300.
22. Philpott SM, Buehring GC. Defective DNA repair in cells with human T-cell leukemia/bovine leukemia viruses: role of tax gene. *J Natl Cancer Inst*. 1999;91:933-942.
23. Baltzell K, Buehring GC, Krishnamurthy S, Kuerer H, Shen HM, Sison JD. Limited evidence of human papillomavirus in breast tissue using molecular in situ methods. *Cancer*. 2012;118:1212-1220.