Human Mammary Tumor Virus, Human Papilloma Virus, and Epstein-Barr Virus Infection Are Associated With Sporadic Breast Cancer Metastasis

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ABSTRACT

BACKGROUND: Viral cause of sporadic breast cancer (SBC) has been suggested based on the experimental murine model of mammary tumor caused by mouse mammary tumor virus (MMTV), Epstein-Barr virus (EBV), and human papillomavirus (HPV). While some studies have demonstrated the presence of viral sequences of MMTV, HPV, and EBV in breast cancer cells, others failed. These contradictions may be attributed to the geographical distribution of breast cancer incidence and/or technical variations. In the current study, we aimed to investigate the correlation of MMTV, HPV, and EBV infections with the development of breast cancer in Jordanian patients.

METHODS: One hundred SBC tissue samples were subjected to laser capture microdissection for the selection of tumor cells populations. Fluorescence polymerase chain reaction (PCR) was used to detect the presence of the MMTV env-like sequences. Real-time PCR was used for HPV and EBV detection, and EBV was further confirmed by chromogen in situ hybridization (CISH).

RESULTS: Mouse mammary tumor virus, HPV, and EBV were detected in SBC in 11%, 21%, and 23%, respectively. Only 3 of 52 (5.7%) positive cases demonstrated multiple virus infections. However, 49 of 52 (94%) of the positive cases revealed the presence of 1 type of viral sequences. Consequently, 52% of the studied breast cancer cases were infected with at least 1 type of the aforementioned viruses.

CONCLUSIONS: The current cohort suggests that MMTV, HPV, and EBV have a potential role in the development of breast cancer and adding more reasons to proceed with the quest of a possible viral origin of breast cancer.

KEYWORDS: Breast cancer metastasis, EBV, HPV, MMTV

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Introduction

Notwithstanding that breast cancer is considered the most common cancer among women worldwide and its cause has been studied for many decades, the precise causes are unclear. Up to 10% of the breast cancer cases are hereditary, as a consequence of the transmission of pathogenic mutations in certain genes such as *BRCA1* and *BRCA2*.[1] The pathogenesis of SBC is largely attributed to estrogens that act as powerful promoting agent,[2] while very limited percentages of the breast cancer cases are attributed to radiation exposure.[3]

Worldwide, at least 1 of 5 cases of cancers has been linked with viral infection.[4] Viral cause of breast cancer was not accepted until the discovery of the mouse mammary tumor virus (MMTV), a retrovirus passed by mothers to offspring during lactation.[5] Later, a shred of evidence demonstrated the presence of the MMTV-env gene sequence (MMTV-egs) in up to 38% of human infiltrating SBC.[6,7] In a cluster of studies, we showed that the presence of MMTV-egs is strictly associated with sporadic breast cancer (SBC) progression,[8] and demonstrated the presence of MMTV in saliva and salivary glands of patients with breast cancer.[9] Recently, we revealed that

MMTV is almost absent in hereditary breast cancer (HBC) compared with 30% of SBC.[10] Even though, the low prevalence of MMTV in the HBC could be involved in exacerbating of breast cancer. On the contrary, different studies have reported contradicting results.[11,12]

Epstein-Barr virus (EBV) is an oncogenic virus that has been linked to different types of cancers, including Burkitt lymphoma, post-transplant lymphoma, nasopharyngeal carcinoma, Hodgkin disease, and possibly other cancer types.[13-16] The causal role of EBV in breast cancer is controversial as well.[17,18] Epstein-Barr virus was reported in breast cancer at different frequencies in different countries. In a study from India, EBV-encoded RNA in situ hybridization (EBER-ISH) was positive in 30.1% of breast cancer cases,[19] while a study from China detected EBV in 60% of the studied breast cancer cases using multiplex polymerase chain reaction (PCR).[20] European studies demonstrated EBV positivity in 25.8% (Portugal), 33.2% (France) of breast tumor using real-time PCR technique.[21] A lower prevalence of EBV (8%) was detected in breast cancer from Iran using real-time PCR.[22] However, others failed to detect EBV in investigated breast cancer cases.[23-25] These controversial results could be attributed to geographical or technical variances or maybe both, for instance, in PCR-based studies, evidence for the presence of EBV exclusively in the breast cancer cells is required to distinguish between cancer cells and infiltrating lymphocytes.

Human papillomavirus (HPV) is widely associated with cervical,[26] head, and neck cancers.[27] However, the etiologic role of HPV in breast cancer remains inconclusive. Few genotypes (16, 18, 33, 39, and 51) of HPV were shown to be associated with breast cancer.[28-31] The HPV genome consists of 3 main segments, long control region (LCR), early region (E), and late region that encode E1, E2, and E4 to E7 proteins. E6 and E7 are both involved in host cell proliferation.[32] Although many studies have demonstrated a lack of association between HPV and breast cancer,[33-37] other studies strongly demonstrated an association between the HPV infection and the development of breast cancer.[31,38,39] These opposing results may be attributed to methodological differences and/or geographical distributions. Herein, we investigated the prevalence of MMTV, HPV, and EBV in breast cancer cases and their association with clinical and pathologic parameters in Jordanian patients.

Materials and Methods

Specimens

One hundred infiltrating ductal SBC were selected randomly from the archive of King Abdullah University Hospital, Al Basheer Hospital, and King Hussein Cancer Center representing most geographical regions in Jordan. The formalin-fixed paraffin-embedded (FFPE) samples were collected and analyzed for the presence of MMTV, HPV, and EBV genomes. Twenty cases of reduction mammoplasty (normal breast tissues) and 30 cases of blood samples from healthy donors were considered as a control group. This study was approved by the Institutional Review Board (IRB) committee of King Hussein cancer center with the IRB number 10KHCC71.

Laser microdissection

Automated laser captured microdissected (Leica Microsystems, Wetzlar, Germany) was used to carefully selecting the tumor cells regions and avoiding the stromal and inflammatory cells. Sections of 5 μm were obtained from each case, mounted on micro-dissecting membrane slides, stained and a total of about 10 000 to 15 000 tumor cells were selected carefully for DNA extraction.

DNA extraction

DNA from microdissected tumor cells were extracted using a homemade lysis buffer containing 0.1 mg/mL proteinase K per sample, 10 mM tris-HCL, 1 mM EDTA, 1% tween 20, pH8. The quality and amount of extracted DNA were evaluated by Epoch-Bioteck. Extracted DNA was then checked for the absence of PCR inhibitors by amplifying GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as a positive control.

The MMTV sequence detection

The MMTV-env-like sequence was detected using the fluorescence-nested PCR technique. The size of fluorescent amplicons was checked on ABI PRISM 3100 sequencer. The first set primer was designed to produce a 248 bp fragment from an MMTV-like env sequence. The sequence of first primers was as follows: forward, 5'-GATGGTATGAAGCAGGATGG-3' labeled with FAM; and reverse, 5' CCTCTTTTCTCT ATATCTATTAGCTGAGGTAATC-3'. The DNA concentration for the first round was 500 ng genomic DNA and 2 μL of the amplicon in the second round. Both positive and negative controls were included in each run. The result was analyzed by capillary electrophoresis that appeared as peaks in an electropherogram. The positive result appears as a peak at 248 bp after the PCR run (Figure 1).

Epstein-Barr virus sequence detection

Real-time PCR was conducted for the qualitative detection of EBV sequence in FFPE of breast cancer. Primers (EBNA2 FCATCGCAGGGTTCTTACCAT, EBNA2 R GAAGAAA CAGCC TCCTGCAC) were designed to amplify the DNA sequence of the single-copy gene encoding the nuclear antigen 1 (EBNA 2). The primers were optimized using DNA extracted from FFPE of Hodgkin lymphoma positive for EBV. Briefly, the final volume was 30 μL containing 15 μL of ready-to-use sybr-green master-mix, 3 μL of extracted DNA, 0.5 μL of each appropriate primer (10 μM), and 11 μL of dH_2O. The PCR was optimized as the following: 95°C activation for 7 minutes, 40 cycles of denaturation at 95°C for 45 seconds, annealing at

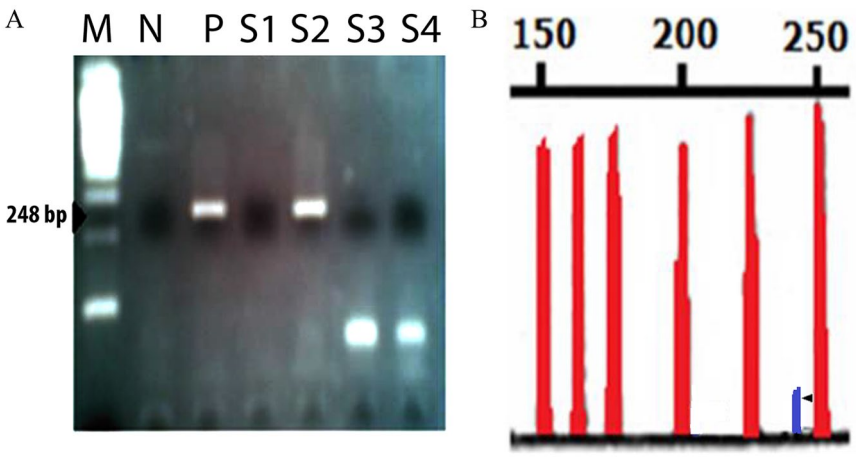

Figure 1. A representative example of MMTV positive cases. (A) Gel electrophoresis shows positive MMTV in SBC samples (left). (B) Capillary electrophoresis of MMTV shows a blue peak on 248 after PCR (right). MMTV indicates mouse mammary tumor virus; PCR, polymerase chain reaction; SBC, sporadic breast cancer.

58°C for 45 seconds and extension at 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes.

Epstein-Barr virus chromogen in situ hybridization

Sections of 4 μm thicknesses were mounted on positively charged slides and incubated overnight at 37°C. Then, the sections were deparaffinized and rehydrated using xylene and an ascending series of ethanol. A tissue pretreatment kit (cytocell) was used to retrieve the antigens and then incubated with proteinase k to get access to the nucleus. Probe for EBER, alkaline phosphatase detection kit from BioGenex was used according to the manufacturer's protocol. The EBER was detected in breast cancer tissue as a nuclear red stain (Figure 1).

Human papillomavirus genotype detection

Genomic DNA was assessed by analysis of the reaction controls (water and HPV positive) amplified using HPV High-Risk Typing Real-TM Kit (Sacace Biotechnologies, Italy) to detect DNA of high-risk HPV in SBC. Real-time amplification procedure was used for qualitative detection and genotyping of the HPV (16,18,31,33,35,39,45,51,52,56,58,59) by multiplex real-time amplification of 4 tubes for each sample according to the manufacturer's instructions using Rotor Gene-Q-Qiagen. Each tube contains primers directed against regions of 3 HPV types and the β-globin gene is used as an internal control. The result was interpreted by a program in Microsoft Excel format provided with the kit according to the manufacturer's instructions. The specificity of the kit is 100% with a sensitivity of not less than 1000 copies/mL.

Results

A total of 100 FFPE breast cancer cases were collected from different Jordanian hospitals, representing various geographical provinces in Jordan. The clinical and pathologic details of the FFPE breast cancer samples are presented in Table 1.

Twenty cases of normal breast samples obtained from reductive mammoplasty and 30 cases from healthy blood donors were used as a control group. Epstein-Barr virus sequence was detected in 24% of the breast cancer cases, while normal breast tissue and blood samples from a healthy donor were positive for EBV in 0 of 20 (0%) and 3 of 30 (10%) cases, respectively ($P = .0065$) (Table 2).

The presence of the EBV was confined to the malignant cells which was confirmed by chromogen in situ hybridization (CISH) assay (Figure 2).

The presence of HPV was considered positive if 1 or more of the high-risk genotypes (16, 18, 31, 35, 39, 45, and 58) were detected. Human papillomavirus was demonstrated in 21 of 100 (21%) (HPV-16: 4 of 100; HPV-18: 10 of 100) of the breast cancer cases compared with 0 of 20 (0%) and 2 of 30 (7%) in normal breast tissue and blood from healthy donors, respectively ($P = .0071$) (Table 2).

The MMTV viral sequence was detected in 11 of 100 (11%) of the studied cases, while the control group did not show any signs of MMTV infection ($P = .016$) (Table 2). The mono and/or multiple infections of EBV, HPV, and MMTV were detected in 52% of breast cancer cases from different Jordanian patients. Multiple viral infections were identified in only 3 of the 52 (5.7%) positive cases. Interestingly, 94% of the positive cases were infected with only one type of viruses. When we correlated the prevalence of EBV, HPV, and MMTV with the clinical and pathologic parameters of SBC, the only significance was found between HPV and age grouping ($P = .039$) (Table 3).

Moreover, univariate analysis showed a significant association between lymph node (LN) metastasis and any viral infection ($P = .029$). These data could contribute to our understanding of the role of HPV, EBV, and MMTV in human breast cancer.

Table 1. Clinicopathologic characteristics of sporadic breast cancer of patients from different provinces of Jordan.

CLINICOPATHOLOGIC CHARACTERISTICS	N (%)
Age (y)	
<40	24 (24%)
40-50	30 (30%)
>50	46 (46%)
Cancer type	
IDC	85 (85%)
DCIS	5 (5%)
ILC	10 (10%)
Grade	
I	12(12%)
II	56(56%)
III	32(32%)
LN metastasis	
Yes	64 (64%)
No	36 (36%)
Lymphatic reaction	
Yes	45 (45%)
No	55 (55%)
IHC	
ER+	66 (66%)
ER–	34 (34%)
PR+	63 (63%)
PR–	37 (37%)
HER2+	29 (29%)
Equivocal	13 (13%)
HER2–	58 (58%)

Abbreviation: LN, lymph node; DCIS, Ductal carcinoma insitu; IDC, Invasive ductal carcinoma; ILC, Invasive lobular carcinoma; IHC; Immunohistochemistry; ER, Estrogen receptor; PR, Progesterone receptor; HER2, Her2 protein.

Discussion

Until now, no definitive consensus has been gained on the association between viral infection and the development of breast cancer, as many investigators have failed to detect the viral genome in breast cancer. Despite the detection of viral sequences in breast cancer tissues, there has been concern regarding whether the sequences could be attributed to non-tumor cells (eg, infiltrating lymphocytes) rather than tumor cells. Low viral load: tumor cell ration (not quantified in this study) is another reason that many researchers dismiss viral causation of breast cancer. However, the low ratios do not justify the conclusion that the viruses are not involved. This low viral ratio might result from selective destruction of the infected tumor cells; asymmetric transfer of viral genome during cellular division may also be another reason of low infected cells in the tumor.[40] Herein, laser microdissection was used to get a reliable and enriched population of tumor cells. We examined the prevalence of EBV, HPV, and MMTV in 100 paraffin-embedded tissue samples from Jordanian breast cancer patients alongside a possible association with their clinical and pathologic parameters.

The role of EBV in breast cancer is unclear in part because of uncertainties over its cell tropisms and the timing of its effects. However, Hu et al[41] showed that EBV can infect the primary mammary cells through CD21 receptors which are involved in the early transformation of mammary epithelial cells to malignancy, and then are no longer required after malignancy. Others stated a correlation between late age primary EBV infection and risk of breast cancer[42] and linked EBV-positive breast cancer with ER-negative, amplified HER2, and aggressiveness.[43] Our results revealed the presence of EBV sequences in 24% of the studied cohort, which is consistent with previous studies from Portugal (25.8%), Pakistan (24.4%), and Eritrea (27.7%). In addition, a higher prevalence of EBV infection was reported in studies from Sudan and Syria where the EBV genome was detected in about 53% and 51.8%, respectively.[44,45] On the contrary, other investigators either failed to detect the viral sequence or detected it at a low percentage. This could be attributed to the geographical distribution or technical challenges such as large mastectomy specimens and consequently the fixation time. In a recent study of transcriptome analysis of EBV-human cell line interaction, the investigators revealed co-expression of tumor suppressor genes like *BRCA1* and oncogenes like CFOS during the lytic phases of EBV replication.[46] These findings might explain the high prevalence of EBV in breast cancer patients with a low frequency of *BRCA1* and *BRCA2* mutations. In the same context, an epigenetic study of *BRCA1, BRCA2*, and *P14*, tumor suppressor genes, in EBV-positive breast cancer revealed hypermethylation and gene silencing of these genes suggesting a potential role of the viral infection in the epigenetic silencing of these tumor suppressor genes.[44] In the current study, the EBV-positive cases were not significantly associated with age, hormonal status, tumor grade, or LN metastasis. However, the EBV-positive cases were noticed to be higher in the age of more than 40 years, high-grade tumors, and lack of HER2 expression.

Human papillomavirus is an oncogenic virus considered being widely associated with endocervical and head and neck cancers. Recently, there is a growing body of evidence suggesting a potential role of HPV infection in the development of breast cancer. In 1992, Di Leonardo et al[47] reported the presence of HPV in breast cancer samples for the first time. In the current study, we detected the HPV viral sequences (the high-risk genotypes) in 21% of breast cancer study cases,

Table 2. The prevalence of EBV, HPV, and MMTV in sporadic breast cancer, normal breast tissues, and blood samples from healthy donors.

	EBV+	EBV–	*P* VALUE	HPV+	HPV–	*P* VALUE	MMTV+	MMTV–	*P* VALUE
Invasive breast cancer	24 (24%)	76 (76%)	.0065	21 (21%)	79 (79%)	.0071	11 (11%)	89 (89%)	.016
Normal breast tissue	0 (0%)	20 (100%)		0 (0%)	20 (100%)		0 (0%)	20 (100%)	
Healthy donor blood	3 (10%)	27 (90%)		2 (7%)	28 (93%)		0 (0%)	30 (100%)	

Abbreviations: EBV, Epstein-Barr virus; HPV, human papillomavirus; MMTV, mouse mammary tumor virus.

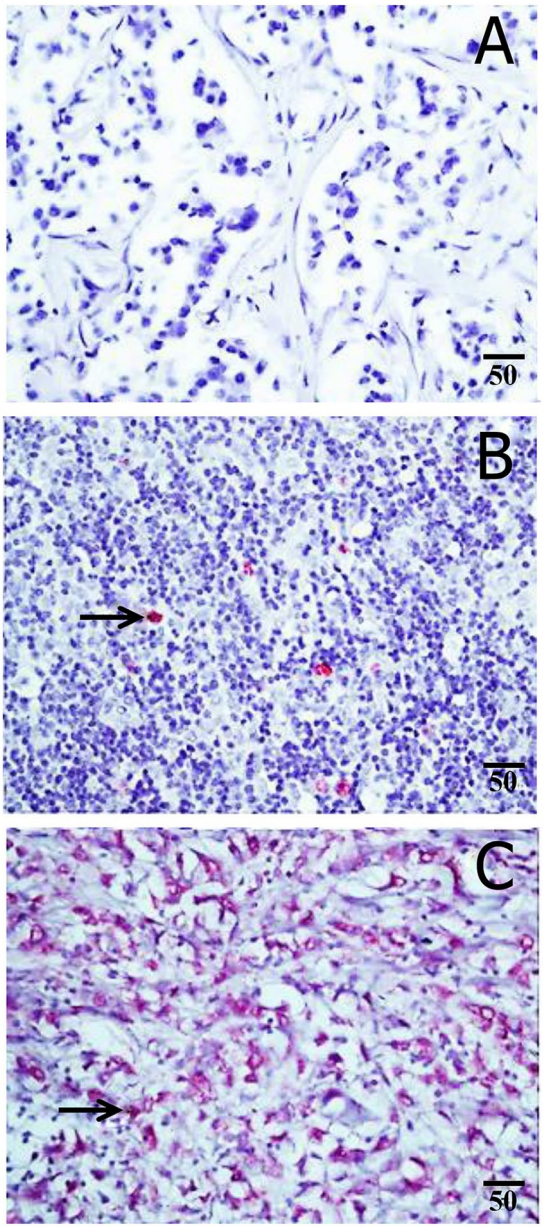

Figure 2. Epstein-Barr virus encoded RNA detected in FFPE tissue stained with fast red by CISH. (A) Negative breast cancer; (B) Positive control cells show red stain (arrow); (C) Invasive breast cancer positive for EBV (arrow). CISH indicates chromogen in situ hybridization; FFPE, formalin-fixed paraffin-embedded.

compared with 4% in the control group. The most prevalent HPV genotypes were HPV-18 (48%) and HPV-16 (19%). Our results were in accordance with Khan et al[30] study 2008 who reported the HPV in 21% of breast cancer cases but is contrary to our results of the HPV-16 (92%) which was the most common genotype. Other investigators have reported a higher prevalence of HPV with different genotypes in breast cancer patients. For instance, a recent study from Iran estimated the prevalence of HPV in breast cancer at 48.6% and the most common genotype was HPV-18 (22.2%).[28] In an Indian study, the HPV was reported at 63.9% and the HPV-16 genotype was found at 69% of the HPV cases.[29] Also, a UK study reported 42% of HPV infection in breast cancer cases and 20% was reported to be HPV-39 genotype.[31] A Mexican study showed 40% of HPV infection with 87.5% of HPV-16 in the tested breast cancer cases,[48] while a study from China demonstrated the prevalence of HPV at 43.8% and HPV-33 at 43.8%.[49] The variations in the prevalence and genotypes of HPV might be attributed to the geographical distributions.

At the molecular level, the *BRCA1* and *BRCA2* tumor suppressor genes were found to be expressed at a low level in HPV+ breast cancer compared with HPV– breast cancer,[28] suggesting a potential role of HPV in interaction with *BRCA1* and *BRCA2*, by decreasing their expression and thus developing breast cancer. Our results showed a significant correlation between HPV+ breast cancer and age (Table 3). On the contrary, no significant correlation was found between HPV+ breast cancer and grade, LN metastasis and ER, PR, and HER2 status. Our findings were in accordance with Naushad et al[50] findings who did not show any significant correlation between the clinicopathologic parameters and HPV infection in breast cancer.

Mouse mammary tumor virus has been suggested as a candidate for the development of breast cancer several decades ago.[51] Since then, cumulative studies favor the causative role of MMTV in the development of breast cancer. Interestingly, the MMTV-like long terminal repeat (LTR) was detected in 41.5% in human breast cancer but not in normal breast tissues[52]; also, the MMTV-env like sequence was detected in high prevalence in ductal carcinoma in situ (82%) compared with their corresponding invasive tumor cells (27%).[8] The viral particles of MMTV were isolated from ascitic fluid of metastatic breast cancer.[53] Moreover, MMTV was shown to infect many human cell types including cells derived from breast cancer.[54] Interestingly, MMTV-env like sequence was detected in the benign breast tissue of Australian patients who develop breast cancer after several years.[55] A previous study revealed the

Table 3. Association of clinicopathologic features with the prevalence of MMTV, EBV, and HPV.

CLINICOPATHOLOGIC CHARACTERISTICS	NUMBER	EBV+ (%)	EBV− (%)	*P* VALUE	HPV+ (%)	HPV− (%)	*P* VALUE	HMTV+ (%)	HMTV− (%)	*P* VALUE	POS-VIRAL INFECTION (%)	NEG-VIRAL INFECTION (%)	*P* VALUE
Age (y)													
<40	24 (24%)	4 (17%)	20 (83%)	.365	4 (17%)	20 (83%)	.039	4 (17%)	20 (83%)	.586	9 (37.5%)	15 (62.5%)	.168
40-50	30 (30%)	6 (20%)	24 (80%)		11 (37%)	19 (63%)		3 (10%)	27 (90%)		19 (63%)	21 (37%)	
>50	46 (46%)	14 (30%)	32 (70%)		6 (13%)	40 (87%)		4 (9%)	42 (91%)		24 (52%)	22 (48%)	
Grade													
I	12 (12%)	3 (25%)	9 (75%)	.776	1 (8%)	11 (92%)	.478	1 (8%)	11 (92%)	.921	5 (42%)	7 (58%)	.166
II	56 (56%)	12 (21.4%)	44 (78.6%)		11 (20%)	45 (80%)		6 (9%)	51 (91%)		26 (46%)	30 (54%)	
III	32 (32%)	9 (28%)	23 (72%)		9 (28%)	23 (72%)		4 (12.5%)	28 (87.5%)		21 (66%)	11 (34%)	
LN metastasis													
Yes	64 (64%)	19 (33%)	43 (67%)	.095	15 (23%)	49 (77%)	.487	8 (12.5%)	56 (87.5%)	.487	38 (59%)	26 (41%)	.02*
No	36 (36%)	5 (14%)	31 (86%)		6 (17%)	30 (83%)		3 (8%)	33 (92%)		14 (39%)	22 (61%)	
ER													
Positive	66 (66%)	16 (24%)	50 (76%)	.937	13 (20%)	53 (80%)	.656	6 (9%)	60 (91%)	.395	33 (50%)	33 (50%)	.327
Negative	34 (34%)	8 (24%)	26 (76%)		8 (24%)	26 (76%)		5 (15%)	29 (85%)		19 (56%)	15 (44%)	
PR													
Positive	63 (63%)	18 (29%)	45 (71%)	.162	11 (17%)	52 (83%)	.257	5 (8%)	58 (92%)	.052	33 (52%)	30 (48%)	.466
Negative	37 (37%)	6 (16%)	31 (84%)		10 (27%)	27 (73%)		6 (16%)	31 (84%)		19 (51%)	18 (50%)	
HER2													
Positive	29 (29%)	7 (24%)	22 (76%)	.316	6 (21%)	23 (79%)	.853	5 (17%)	24 (83%)	.248	16 (55%)	13 (45%)	.068
Equivocal	13 (13%)	1 (8%)	12 (92%)		2 (15%)	11 (85%)		0 (0%)	13 (100%)		3 (23%)	10 (77%)	
Negative	58 (58%)	16 (27%)	42 (73%)		13 (22%)	45 (78%)		6 (10%)	52 (90%)		33 (57%)	25 (43%)	

Abbreviations: EBV, Epstein-Barr virus; HMTV; human mammary tumor virus; HPV, human papillomavirus; LN, lymph node ER, Estrogen receptor; PR, Progesterone receptor; HER2, Her2 protein.

presence of MMTV-env like sequence (57% DNA and 34% RNA) in the saliva of breast cancer patients.[9] Recently, we demonstrated that HBC with *BRCA1* and *BRCA2* mutations displayed a very low level of MMTV-env like sequence (4%) compared with the SBC (30%).[10] In this study, we demonstrated a low prevalence of MMTV-env like sequence (11%) in SBC of Jordanian patients. In the same context, a study from the eastern part of Saudi Arabia found a low prevalence of MMTV-env like sequence (6%) in the breast cancer tissues.[56] Geographical distributions and other pathologic factors such as *BRCA1* and *BRCA2* expressions, and mutation status might explain the variations in the prevalence of MMTV in breast cancer. Interestingly, our results showed that 52% of the breast cancer cases studied were positive for at least 1 of the studied viruses; this percent could be increased if other viruses like cytomegalovirus and leukemia virus are included. However, 3 cases revealed multiple viral infections. The mono/multiple viral infections were found to be significantly correlated with LN metastasis ($P = .029$) (Table 3).

Conclusions

Our findings underscored the possible role of MMTV, EBV, and HPV in the development of human breast cancer in the Jordanian population. Besides, the current results demonstrated a significant association between a viral infection and LN metastasis of breast cancer.

Author's Note

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Author Contributions

MAAl-H contributed to the study conception and design. Specimen collection and cases reviewing were performed by IM, MAl-H, and MS. Molecular techniques and data interpretations were done by MSAl-Z, IA, RK, SJ, and CMM. ADAl-N performed the statistical analysis. The first draft of the manuscript was written by MAAl-H and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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