RESEARCH ARTICLE

Detection of Epstein Barr Nuclear Antigen-1 (EBNA-1), Early Antigen 1F, 2R (EA-1F, EA- 2R) along with Epstein–Barr virus Latent Membrane Protein 1 (LMP1) in Breast Cancer of Northern India: An Interim Analysis

Vanshika Shahi[1], Preeti Agarwal[1]*, Sumaira Qayoom[1], Vijay Kumar[2], Shikha Tewari[3], Shivanjali Raghuvanshi[1], Uma Shankar Singh[1], Madhu Mati Goel[3]

Abstract

   Introduction: Worldwide, breast cancer (BC) is a prominent cause of death, with a disproportionately high incidence in developed countries. Epstein-Barr virus (EBV) infection has been reported in up to 90% of the world's population. Although the exact link of EBV infection and breast carcinoma is not yet determined. The present study was carried out to assess the pathological correlation of EBV infection and BC in women from Northern India. **Methodology:** In this prospective observational study, 130 patients with histologically proven breast carcinoma were included. After detailed histology, the paraffin block with infiltrative tumor was selected for molecular analysis and further immunohistochemistry (IHC)– EBV PCR and Epstein–Barr virus latent membrane protein 1 (LMP1) IHC. **Results:** Most of the patients were diagnosed with Infiltrating Ductal Carcinoma not otherwise specified (IDC-NOS), followed by Infiltrating Ductal Carcinoma + Ductal Carcinoma in situ (IDC + DCIS). The total of 25 tissues of breast carcinoma had positive EBV PCR results (19.23%). The co-relation between the molecular and immunohistochemical results was significant in 11/25 cases that showed immunoexpression for LMP1 by IHC. Sensitivity of 44% and specificity of 100% were observed for LMP1 IHC, having a PPV value of 100% and an NPV of 88%. No significant correlation was observed between age, tumor subtype, grade, stage with respect to EBV infection; however, there was a significant association with nodal metastasis with extra nodal extension in tumors that had EBV infection. **Conclusion:** The present study establishes an association between LMP1 and patients with EBV positive breast cancer. The authors suggest that additional multicentric studies be conducted to strengthen the reliability and generalizability of the observations of the current study.

   Keywords: EBV- breast cancer- clinico-pathological variables- LMP1- viral oncogenesis

Introduction

   Worldwide, breast cancer (BC) is a prominent cause of death, with a disproportionately high occurrence in developed countries (Yahia et al., 2014; Pai et al., 2018). A wide range of biological characteristics make it a malignancy with diverse clinical outcomes. Multiple pathogenesis have been implicated. Conventional risk factors such as gender, family history, obesity, physical inactivity, advanced age, and nulliparity, as well as alcohol intake, account for a percentage of cases (Reza et al., 2015; Su et al., 2020).

   Viral infection has been associated with a multitude of malignancies, including Burkitt lymphoma (BL), nasopharyngeal carcinoma (NPC), post-transplant lymphomas, oral cancers and Hodgkin disease (Labrecque

et al., 1995; Sarac et al., 2001; Serraino et al., 2001;Shahi et al., 2021). However, it was not until 1995 that a link could be established between Epstein-Barr Virus (EBV) infection and BC. Epstein-Barr virus (EBV) infection has been reported in up to over 90% of the world's population. The exact nature of EBV infection and breast carcinoma could not be determined due to conflicting and contrasting results. Numerous studies have shown that between 30 and 50% of breast tumors tested positive for EBV (Xue et al., 2003; Fawzy et al., 2008; Joshi et al., 2009; Lorenzetti et al., 2010; Glenn et al., 2012). At the same time, EBV was not identified in other investigations (Chu et al., 1998; Deshpande et al., 2002; Khoury et al., 2013) A variety of factors could explain the inconsistent results found in the literature, including differences in the prevalence of EBV in different regions, differences in the platforms used to

[1]Department of Pathology, KGMU, Lucknow, UP, India. [2]Department of Surgical Oncology, KGMU, Lucknow, UP, India. [3]Department of Laboratory Medicine and Pathology, Medanta Hospital, Lucknow, UP, India. *For Correspondence: preavn@gmail.com

detect viral genome across studies, technical difficulties in demonstrating virus presence in tumor cells, and so on (Huo et al., 2012).

Comprehension of tumor biology is imperative, as it not only leads to better prediction of disease behaviour, but also guides treatment plan and patient care. Scientists around the world have focused their attention on the possibility that EBV may be associated with BC, as it may provide a preventive strategy for cancer control. Research in western countries has found a relationship between EBV and BC. However, there is a dearth of research from India (Joshi et al., 2009). Established risk factors for BC can only explain a small percentage of cases in India, making it necessary to look for additional etiological factors. Due to the ongoing debates over the link between EBV and BC, the scarcity of research from India, and the possibility of more demanding preventive and therapeutic techniques, is a matter of concern. In view of the above background, the present study was carried out to assess the pathological correlation of EBV infection and BC in the northern India population. We also attempted to compare the results of EBV-PCR with respect to Epstein–Barr virus latent membrane protein 1 (LMP1) immunohistochemistry (IHC).

Materials and Methods

This was a prospective observational study of 130 patients with histologically proven breast carcinoma diagnosed between 2018-19. All cases with insufficient tissue for molecular study and immunohistochemistry, post chemotherapy/ radiotherapy were excluded (Huo et al., 2012). Ethical clearance from institutional ethics committee was obtained via letter number 1532/Ethics/19. After detailed histology, the paraffin block with at infiltrative tumor in section was selected for molecular analysis and immunohistochemistry.

Molecular testing
DNA extraction was performed from 7-8, 10 micron thick tissue sections from formalin-fixed paraffin-embedded (FFPE) blocks. QIAamp DNA FFPE Tissue Minikit (Qiagen GmbH Hilden, Germany) was used. Subsequently, the extracted DNA was preserved at -20°C.

EBV was detected by PCR using specifically manufactured primers for QP1 and QP2 (Detection of Epstein Barr Nuclear Antigen (EBNA-1) area; product size 213 bp) and Early Antigen 1F, 2R (EA-1F, EA- 2R) (BHRF-1 region; product size 208 bp) of EBV, whereas primers for the beta-globin gene TAL57 region (product size 286 bp) were used as housekeeping. Samples with a high CT value were excluded from the experiment.

Ten μlPowerSYBR® Green PCR Master Mix (Applied Biosystems by Thermo Fisher Scientific, Warrington, UK), 1 μl each of the synthesised forward and reverse primers (mentioned above), 2 μl of nuclease free water and 6 μl of extracted DNA were used for reaction. Using the StepOne TM 48-well, samples were analysed (Applied Biosystems, Singapore). The PCR products were electrophoresed in a gel with 2 percent agarose to verify their identity (Agarose Multi-Purpose; Roche). To capture images, the Geldoc 2000 system (Bio-Rad) was used. PCR-grade water and no template DNA controls were used to monitor contamination.

The highly conserved EBV regions EBNA-1 and BHRF-1 were amplified to minimize false negative results and increase detection rates. EBV-1, the only virus protein that is constantly expressed in EBV-infected cells, is essential for replication and genome preservation. Similar to Bcl-2, BHRF-1 has anti-apoptotic properties and contributes to cell survival during lytic infection. Two distinct PCR cycles were used to identify EBV: one with three repeats at 40 seconds for each step, and the other with 40 repeats at 30 seconds for each step.

Immunohistochemistry (IHC)
Immunohistochemistry was performed on 3-5 micron FFPE tissue sections. Antigen recovery was performed by heating the sections at 110 ° C for 20 minutes in a recovery buffer in a decloaking chamber (high pH). The primary antibody used was LMP1 (Dako FLEX monoclonal mouse anti-Epstein-Barr virus, LMP clones CS 1-4) and the secondary antibody was Dako REAL EnVDetectSysPerox/ DAB+ Rb/M with DAB as chromogen. The lymph node section of Hodgkin lymphoma was taken as an external control with each run [Figure 1a]. LMP-1 expression was seen both in cytoplasm and nucleus of tumor cells. The following grading was used: 0 = no stained tumour cells, 1 = 1-25% stained tumour cells, 2 = 25%-50% stained tumour cells, and 3 = >51 percent stained tumour cells. Cells without staining were considered negative. (Sarac et al., 2001)

Statistical Analysis
An MS EXCEL spreadsheet was used for data entry; SPSS version 16.0 was used for analysis. The Chi-Square test /Fisher's exact test were used to compare the study variables. For this study, the significance level was set at 0.05.

Results

Total 130 females were enrolled after informed consent. The demographic variables of the study population are summarised in Table 1. The majority of patients were diagnosed with IDC NOS, followed by IDC + DCIS. There was a single case of IDC with mucinous component and one with neuroendocrine differentiation. Carcinoma of the left breast was more frequent in our study group with grade 2 tumors. Modified radical mastectomy was the most frequent procedure performed. Nodal involvement was more often seen. Surprisingly, among luminal classification, TNBC was more common 67/103 (51.54%) in our population followed by Her2 enriched (when only 3+ was considered positive), luminal B and luminal A, respectively (Table 2). As in our set-up follow up FISH for Her2 2+ cases were not available at the time of the study it was not performed. The 25 tissues of breast carcinoma had positive EBV PCR results (19.23%) while all the rest were negative. The correlation between molecular and immunohistochemical results

Table 1. Clinico-Demographical Profile of EBV Patients

			EBV Negetive [n=105]		EBV Positive [n=25]		P-value
			N	%	N	%	
Age (Mean±SD)			46.31 ± 13.33		48.24 ± 11.34		t=0.6681
							p=0.5052
Diagnosis	IDC NOS		89	84.76%	21	84.00%	X=4.516
	IDC + DCIS		15	14.29%	3	12.00%	P=0.2109
	IDC + Mucinous component		1	0.95%	0	0.00%	
	IDC + neuroendocrine		0	0.00%	1	4.00%	
Side	Right		46	43.81%	10	40.00%	X=0.1195
	Left		59	56.19%	15	60.00%	P=0.7296
Grade	1		24	22.86%	5	20.00%	X=1.765
	2		67	63.81%	14	56.00%	P=0.4138
	3		14	13.33%	6	24.00%	
Operation	MRM		95	90.48%	23	92.00%	X=6.494
	Simple Mastectomy		9	8.57%	0	0.00%	P=0.0389*
	Lumpectomy		1	0.95%	2	8.00%	
Tumor	1		16	15.24%	1	4.00%	X=3.588
	2		68	64.76%	21	84.00%	P=0.3095
	3		13	12.38%	2	8.00%	
	4		7	6.67%	1	4.00%	
Node	0		48	45.71%	7	28.00%	X=13.44
	1		41	39.05%	11	44.00%	P=0.0038*
	2		12	11.43%	1	4.00%	
	3		4	3.81%	6	24.00%	
ENE	No		69	65.71%	11	44.00%	X=4.023
	Yes		36	34.29%	14	56.00%	P=0.0449*
ER	No		87	82.86%	19	76.00%	X=0.6307
	Yes		18	17.14%	6	24.00%	P=0.4271
PR	No		88	83.81%	19	76.00%	X=0.8457
	Yes		17	16.19%	6	24.00%	P=0.3578
Her2neu	-ive	0	54	51.43%	12	48.00%	X=0.9455
		1+	2	1.90%	0	0.00%	P=0.8144
		2+	15	14.29%	3	12.00%	
	+ive	3+	34	32.38%	10	40.00%	
ki67			16.361±13.758		20.84±17.372		t=1.388
							p=0.1677
LMP Intensity	-ive	0	105	100%	14	56.00%	
	+ive	1	0	0%	4	16.00%	X=50.47
		2	0	0%	5	20.00%	P<0.0001*
		3	0	0%	2	8.00%	

Table 2. Molecular Classification of Cases

Molecular classification	Numbers	Percentages
Luminal A	7	5.38%
Luminal B	20	15.38%
Triple Negative	67	51.54%
Her 2 enriched	36	27.69%

was significant; 11 of 25 cases positive for PCR showed positive immunoexpression for LMP1 by IHC (Figure 1). While analyzing the diagnostic value of LMP intensity over EBV results, the sensitivity of 44% and specificity of 100% were observed, having PPV value of 100% and NPV of 88% (Table 3).

While comparing the EBV results with molecular classification, age, tumor size, grade and stage no significant results were seen. However with respect
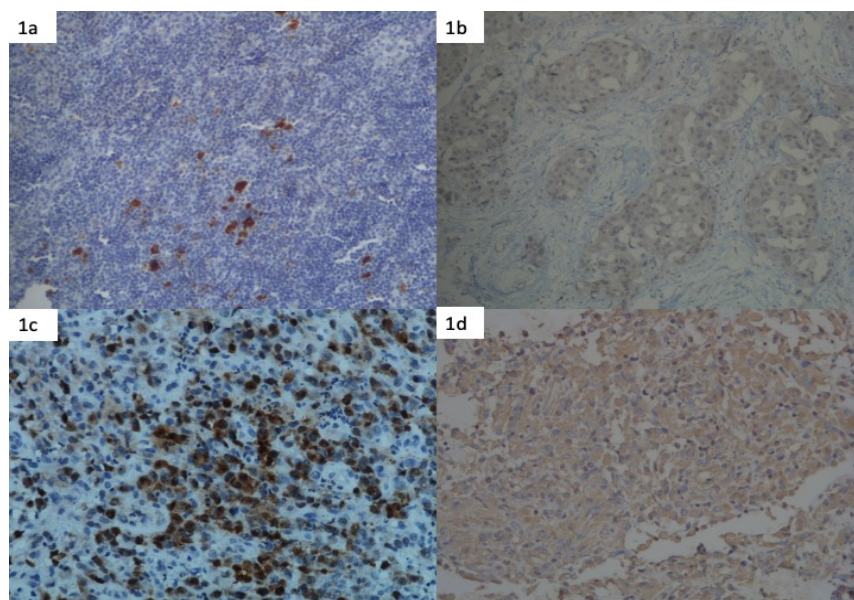
Figure 1. Immunohistochemistry Image Collage of the Study: The control result of LMP1 in the Hodgkin lymphoma case is seen in 1a, weak cytoplasmic expression is seen in 1b, strong expression is seen in 1c with moderate in 1d (IHCx200)

Table 3. Diagnostic Analysis of LMP Intensity in Patients with EBV

Diagnostic Value	Results		Predictive values	
	Positive	Negative	Sensitivity	0.44
Positive	11 TP	0 FP	Specificity	1
Negative	14 FN	105 TN	PPV	1
P- Value	P<0.0001*		NPV	0.8824

to nodal metastasis and extra nodal extension we saw that EBV infection was more frequently seen in node positive tumor as compared to node negative (X=13.44; P=0.0038) and in tumors displaying extra nodal extension (X=4.023;P=0.0449) .

Discussion

Epstein-Barr Virus, is a common gamma human herpes virus. It is classified as a class I carcinogen by the International Agency for Research on Cancer, and its role in the pathogenesis of a variety of cancers, including NPC, gastric cancer, BL, Non-Hodgkin lymphoma, and Hodgkin lymphoma, is well documented in the literature (Yahia et al., 2014). PCR is a highly sensitive and specific technique for detecting EBV DNA. Majority of studies demonstrating a positive association between EBV and carcinogenesis have used it. The major disadvantage of PCR is that it cannot localise the viral genome, i.e. it cannot distinguish EBV in tumor cells from EBV in surrounding lymphocytes, and there is also a risk of contamination with laboratory EBV (Glaser et al., 1998; Glaser et al., 2004; Baltzell et al., 2012). To circumvent these PCR constraints, laser capture microdissection (LCM), immunohistochemistry (IHC), in situ hybridization (ISH) and Southern blot hybridization with variable sensitivity and specificity may be used. Numerous studies have used multiple tests or different methodologies for EBV detection purposes, resulting in disparate results (Chu et al., 1998; Glaser et al., 1998; Bonnet et al., 1999; Deshpande et al., 2002; Glaser et al., 2004; Baltzell et al., 2012; Khoury et al., 2013).

LCM allows for the separation of tumor cells from surrounding lymphocytes prior to PCR and confirms the localization of the viral genome to tumor cells (Murray et al., 2003). Southern Blot technique is capable of semi-quantitative analysis of viral load; it is less sensitive than PCR (Glaser et al., 1998). The ISH approach for EBER enables direct imaging of viral transcripts within tumour cells, has high sensitivity and specificity, and is widely used to identify EBV infection in a variety of different malignancies. IHC is a widely used and straightforward approach for EBV detection since it allows direct viewing of viral proteins within tumour cells; however, the antibodies' cross-reactivity questions its specificity (Luqmani and Shousha, 1995; Brink et al., 2000; Fina et al., 2001; Chu et al., 2001; Mohammadizadeh et al., 2014).

In the present study, we studied the prevalence of EBV using PCR and IHC methods in breast cancer in females presenting in our setup. Taking into account the advantages and disadvantages of each technique we chose these methods to localise the EBV in Breast cancer cells. Moreover, IHC is widely available in Indian setups, cheaper and can be easily standardised. We also tried to co-relate our results with histomorphological prognostic and predictive variables to look whether EBV infection influences the tumor microenvironment at any level or not. In the present study, we observed that of 130 patients, 105

(80.77%) patients were EBV negative and 25 (19.23%) were EBV positive by PCR. On IHC 44% of the PCR positive cases harboured EBV in tumor cells. However, the specificity of immunohistochemical results with respect to PCR was one hundred percent. Upon assessing the diagnostic value of LMP intensity with respect to the EBV PCR results, we found a sensitivity of 44%, with a PPV value of 100% and an NPV of 88%.

Mohammadizadeh et al., (2014) used the IHC technique to examine the expression of the EBV LMP-1 antigen in invasive breast cancer and adjacent normal breast tissue. Our study relies on it. They identified LMP-1 expression in 7.5 % of the breast cancer cells tested, but none in normal breast tissue samples close to the tumour. The present study confirmed this finding, and identified similar prevalence of LMP1 in breast cancer cells 11/130 (8.46%) though the PCR positivity was just double 19.23% (25/130) the phenomenon may be explained by the reasons discussed above.

The detection of EBV in the study is consistent with a previous study from India by Pai et al. 2016, in which the authors found an association with EBV in approximately 55% (28/51) of cases of BC using IHC targeting the EBNA1 antigen (Pai et al., 2017). These findings are consistent with the findings of a meta-analysis involving 24 studies involving 1535 cases in which approximately 29.3 % of BC cases were infected with EBV, and geographical variation in the prevalence of EBV was demonstrated; the highest prevalence was found in Asia (35.25 %), and the lowest prevalence was found in the United States of America (18.27 %). While this study focused on LMP1, we also found approximately 44% of LMP1 positive cases by IHC among EBV positive breast cancer cases (Huo et al., 2012).

Joshi et al., (2009) from India investigated the presence of EBV nuclear antigen-1 (EBNA-1) in breast cancer tissue and benign breast disease in rural Indian women. They found significant expression of EBNA-1 in breast cancer tissue compared to benign breast diseases, with a higher immunological response against EBNA-1 in breast cancer patients.

El-Naby et al., (2017) studied both positive EBNA-1 gene and LMP-1 using PCR and IHC on the same sample separately; and found positive results in 10/42 (23.81%) of breast IDC and 6/42 (14.29%) of breast fibro-adenomas (p=0.04). Similarly, we also observed a significant association between LMP1 and patients with EBV-positive breast cancer. Moreover, only nodal involvement indicated a statistically significant association (p=0.003) with EBV presence in malignant tissue in our dataset. Arbach et al., (2006) detected node involvement in 58.3% of their cases.

Yahia et al., (2014) identified the EBV genome in 49 (53.3%) and 10 (11%) cases, respectively, using LMP-1 and EBNA-4. Both LMP-1 and EBNA-4 primers detected a statistically significant difference between cancer tissues and controls (p = 0.0001). They concluded that EBV is strongly associated with breast cancer and that significant epigenetic silencing of tumor suppressors occurs as a result of or in association with viral oncogenesis.

EBV genome signals were detected in a total of 40 samples (27.77%) compared to controls (p = 0.01) with a higher sensitivity when using the EBER primers by Fessahaye et al., (2017). Five out of the 14 samples stained by EBER-ISH, 35.71% were positive for the virus indicating the presence of the viral genome within the tumor cells. Of those stained for IHC, 7 (15.55%) were positive for LMP2, showing a low frequency of viral proteins. Zekri et al., (2012) also concluded that EBV may operate as a promoter of PIBC formation and may contribute to tumour aggressiveness. Our study corroborates with Yahia et al., 2014; Fessahaye et al. 2017 and Zekri et al., 2012 supporting the association of EBV with BC.

In contrast to the above findings, Kadivar et al., (2011) did not observe detectable EBV-DNA in any of the 100 breast cancer samples or 42 control tissues. Their findings suggested that EBV is unlikely to be a substantial factor in the development of breast cancer in Iranian women. Furthermore, Khan et al., (2011) found no correlation between the presence of EBV in infiltrating lymphocytes and morphologic variables and the expression of ER, PR, and HER2.

Plausible explanation for the contradictory findings of the literature could be the geographic variation in the incidence of EBV infections, the variation in the methodologies used, and the different EBV-derived proteins or nucleic acids targeted for viral genome detection, in the subset of BC studied. The type of tissue sample used, for example, FFPE vs. frozen tissue, also influences the results (Huo et al., 2014). Evaluation for EBV detection using a variety of techniques such as polymerase chain reaction (PCR), laser capture microdissection (LCM), immunohistochemistry (IHC), in situ hybridization (ISH), and Southern blot hybridization with variable sensitivity and specificity is one of the main reasons for the conflicting results (Glaser et al., 1998). It is worth noting that the bulk of studies employed ISH as a confirmatory test to verify PCR positive results (Luqmani and Shousha ,1995; Bonnet et al., 1999; Brink et al., 2000; Zekri et al., 2012). In contrast, just a few studies used ISH as the primary detection method (Glaser et al., 1998; Khan et al., 2011).

As far as molecular characterization is concerned, the majority of cases were triple negative (51.54%) followed by Her2neu enriched (27.69%), Luminal B (15.38%) and Luminal A (5.38%). The above is quite contradictory of western data, though a high percentage of TNBC is seen in Indian females (Perrigoue et al., 2005; Hachana et al., 2011; Bagga et al., 2021; Agarwal et al., 2022). Inclusion of samples with no prior chemotherapy or radiation therapy could be a variable for spurious collection of TNBC in the present study group. When comparing the EBV results with molecular classification, no significant findings were observed.

Several previous studies have also examined the association between EBV expression and clinicopathological prognostic factors for breast cancer. Mohammadizadeh et al., (2014) revealed no statistically significant correlation between LMP-1

expression and age, tumor size, tumor grade, and lymph node status on the other. Preciado et al., (2005) did not demonstrate a statistically significant association between EBV expression and poorer clinicopathological factors. Labrecque et al., (1995) found no statistically significant relationship between EBV expression and tumor subtype. According to Hachana et al., (2011) there was no association between EBV expression and patient age, tumour grade, tumour size, or lymph node status. However, they did find a statistically significant correlation between EBV expression and oestrogen receptor negativity. Additionally, Chu et al., (2001) also found that EBV detection was not related to tumor size, grade, or nodal status.

On the other hand, other investigations have indicated a substantial correlation between EBV expression and several clinicopathological prognostic factors for breast cancer. Mazouni et al., (2011) observed that breast tumours with EBV expression had more aggressive characteristics, including increased oestrogen receptor negativity and a high histological grade. According to Fawzy et al., (2008) findings, breast tumours with EBV-DNA showed involvement of more than three lymph nodes. Our results show that EBV expression was not significantly related to tumor size, grade, patients age or hormonal status, but we found that EBV positive tumors had significantly more nodal metastasis as compared to EBV negative tumors with significant extra nodal extension.

According to Glenn et al., (2012) EBV positivity in breast cancer is related to the patient's young age at diagnosis. In terms of tumor subtype, they saw the strongest correlation between lobular carcinoma and EBV infection. Due to the small sample size of the study with respect to tumor subtypes other than invasive ductal carcinoma, we were unable to obtain statistically meaningful data on the link between LMP-1 expression and tumor subtype in invasive breast carcinomas.

Controversial findings from studies on the EBV-breast cancer association underscore the importance of conducting additional research in this sector to obtain more reliable results. Serological studies to determine lifetime exposure to EBV in newly diagnosed cases of breast cancer, as well as concurrent analyses of EBV markers in breast carcinoma tissue from seropositive individuals, can help us further decipher the complex topic of EBV and breast cancer. The present study establishes an association between LMP1 and patients with EBV-positive breast cancer in northern Indian females, though the prevalence is low. The study's shortcomings, however, included small sample size and a single-centric design. India being a country of varied ethnic and geographical variables, the author suggests that additional multicentric studies be conducted to strengthen the reliability and generalizability of the current study's observations.

In conclusions, BC is one of the leading causes of death worldwide, occurring at an alarmingly high rate in developed countries. Up to 90% of the world's population has been found to be infected with EBV. The current investigation demonstrated an association between LMP1 and breast cancer in patients with EBV PCR positive. Additional multicentric investigations, might be conducted to strengthen the reliability and generalizability of the current study findings.

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