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Detection of Epstein-Barr virus (EBV) in women with breast cancer in Iraq using in-situ hybridization and immunohistochemical techniques

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Abstract

The Epstein–Barr virus (EBV) has recently been identified in human breast cancer globally, potentially contributing to the initiation and progression of this malignancy, as well as gastric cancers, nasopharyngeal carcinoma, and bladder cancer, and has been newly associated with breast cancer. The study aimed to detect the presence of Epstein-Barr Virus Nuclear Antigen-1 (EBNA-1) and encoded RNA (EBER) with tumors of the breast in a group of Iraqi women diagnosed with invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) and to correlate expression results with the age of the participants and with the grade, type, size and others clinicopathological finding of breast cancer. A total of 50 formalin-fixed paraffin-embedded tissues from invasive ductal carcinoma (IDC) (92%) and invasive lobular carcinoma (ILC) (8%) biopsy samples constituted the case group, while 30 formalin-fixed paraffin-embedded tissues from non-cancerous breast tissue served as the control group. The presence of Epstein-Barr virus protein (EBER) in breast tissue was assessed using immunohistochemistry (IHC) and chromogenic in situ hybridization (CISH) methods. EBER RNA signals were identified in 31 cases (62%). In the control group, EBER RNA signals were identified in 3 (10%) of the subjects. Significant differences ($P<0.04$) were seen between the research groups for EBV EBER RNA positive signals. The study demonstrated that immunohistochemistry revealed nuclear brown staining in 34 (68%) breast cancer patients. 3 (10%) in the control group. Substantial variations ($P<0.03$) were seen between the study groups concerning the nuclear brown staining of tumor cell signals. IHC and CISH were identified as sensitive techniques for the identification of EBV. The study concludes that EBNA-1 and EBV EBER RNA were overexpressed in our population group.

Keywords: Breast cancer, EBV DNA, CISH, IHC

Introduction

When it comes to female cancers, breast cancer is far and away the most frequent and the top killer [1]. The latest statistics show that both the incidence and death rates will keep going up [2]. To have a good chance of surviving breast cancer treatment, early detection is crucial [3]. Potential risk factors for this malignancy include advanced age, early menarche, late menopause, hormone replacement treatment, and positive family history [4]. The illness is

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considered sporadic as up to 80% of people with breast cancer do not have any known risk factors [4]. Therefore, it is critical to study additional risk factors as the aetiology of most breast cancer patients is unclear. The onco-modulation and carcinogenesis of breast cancer have been associated with infectious microorganisms in recent research [5]. It wasn't until 1964 that scientists found the first signs that viruses may cause cancer in humans [6]. Multiple mechanisms have been linked to tumors caused by viruses since then. We know of seven oncogenic viruses in humans: HTLV-1, HPV, EBV, KSHV, MCPyV, and HBV [6,7]. Breast cancer may be caused by oncogenic viruses in humans and animals, including MMTV, BLV, HPV, and EBV [8]. Because vaccines and antivirals are so effective in reducing viral tumors, virus-induced malignancies provide diagnostic and therapy opportunities [9]. Recent decades have seen an uptick in interest in the common and ubiquitous human cytomegalovirus (HCMV) and its close relative, Epstein-Barr virus (EBV), due to findings linking HCMV to a number of cancers, including breast cancer. Because HCMV is present in a wide variety of tumor types, it might be a therapeutic target for breast cancer and other HCMV-positive malignancies. The addition of valganciclovir to standard treatment enhances glioblastoma survival, according to retrospective clinical studies [10,11]. These individuals also respond favorably to dendritic cell immunization [12].

EBV stands for "human herpes virus 4" and is the only naturally occurring gamma-herpes virus. No sources of animal waste. Anthony Epstein and Yvonne Barr discovered and documented the virus in 1964 in Denis Burkitt's lymphoma tissues; the virus was subsequently named after them [13]. Around 95% of persons in the 35–40 age bracket have contracted EBV, and the majority of people will. Human neoplasms associated with EBV include tonsil, salivary gland, thymus, and female genital tract cancers, in addition to endemic Burkitt lymphoma and undifferentiated nasopharyngeal carcinoma (NPC). Cervical cancer may be influenced by EBV. In 1993, a hitherto unrecognized association between EBV and cervical squamous cell carcinoma was proposed by Landers et al. There is new information that shows how viruses may cause autoimmune disorders such as MS, rheumatoid arthritis, dermatomyositis, Sjögren's syndrome, and systemic lupus erythematosus. One of EBV's many functions is to convert healthy host cells—including those in the immune system—into cancerous ones. It has the potential to alter cells in vitro and is the most powerful oncogenic virus. The mechanisms of B cell transition remain unknown [14]. The virus infects B lymphocytes via surface molecules known as CD21 and HLA class II. These molecules work together to wake up latent B lymphocytes, keep them proliferating indefinitely, and stop them from dividing [15]. There is no viral production in these dormant infected cells, but the EBV genome is retained as a multicopy episome. Only a few viral genes are expressed by these cells: There are six Epstein-Barr virus nuclear antigens (EBNAs): LP, 3A, 3B, and 3C; three latent membrane proteins (LMPs); and six EBNAs that are involved in proliferation via Notch signaling [16]. Proliferation is activated, and apoptosis is blocked by LMP1. Cell transformation is facilitated by two short RNAs (EBERs). After infecting B cells, the virus stays dormant. Aggressive EBV-linked cancers may develop in immunocompromised people; however, in healthy hosts, the immune system regulates the number of EBV-positive cells via the production of memory virus-specific cells and the initiation of B and T-cell responses. Infectious mononucleosis (IM) is a condition that may be transmitted sexually in postpubescent youth by the Epstein-Barr virus (EBV) [17]. It was discovered in 1986 and 1991 that cervical cells could reproduce EBV genomes [18,19]. Different EBV types are characterized by variations in the EBNA2 and EBNA3 genes. Lymphocyte transformation is reduced by type 2 EBV [20]. Infectious agents of both types may coexist. Like other herpes viruses, EBV has two

stages: latent and lytic replication. The virus attaches to host genomic chromatin utilizing EBNA1 and forms a circular episome in the nucleus during the latent phase. Different cell types have different latent gene expression patterns; however, even while latent, the virus still expresses a tiny proportion of its genes and non-coding RNAs. The viral genome must be passively replicated and passed on to daughter cells by the usual processes of cellular division, such as the host DNA replication machinery, for the virus to enter latency. When it comes to people, EBV stays dormant for life, only popping back up every so often to reactivate and replicate lytically. Upon reactivation, the virus enters the lytic cycle, where it replicates and generates new, infectious viruses. When viruses replicate lyticly, they destroy cells and produce infectious viral particles that may infect additional hosts or innocent cells. Additionally, the virus is unable to reproduce, although it can express a subset of lytic genes [21].

Study objectives:

Using immunohistochemistry and chromogenic in situ hybridisation, we will identify EBV DNA in malignant breast tissues and compare the findings to normal breast tissues to determine whether EBV is involved in the evolution of breast cancer. Furthermore, new approaches to prevention or treatment may emerge from a better understanding of the age ranges in which Iraqi women are most likely to get breast cancer.

Patients and methods:

This research used immunohistochemistry and in situ hybridization to look for cases of breast cancer in women from southern Iraq where the EBV human herpes virus was found. Eighty breast tissue blocks, carefully chosen for their archival quality, were processed and fixed using enhanced methods. The blocks were encased in paraffin and included the following groups:

- 50 blocks of breast carcinomas as a case study.
- 30 blocks of non-malignant breast tissues as a control of the study.

Regular slides were used to cut four (4) µm thick sections. Certification pathologists reviewed newly manufactured hematoxylin and eosin (H&E) stained slides and validated the diagnosis of cancer kinds and grades according to the World Health Organization (WHO) criteria. All relevant histological slides were re-examined. To identify EBV DNA using an in-situ hybridization method and Immunohistochemistry (IHC) approach, two additional sections, each 4 µm thick, were cut on positively charged slides.

Preparatory steps

The instructions for the ISH test, which is part of the ZytoVision DNA probe hybridization/Detection system in situ kit, were found in the product brochure.

1- Tissue slices embedded in paraffin were sliced to a thickness of 4-6 µm, floated in a water bath devoid of proteins, and thereafter set on positively charged slides that had been treated with appropriate slide adhesive.
2- Important note: Tissue slices were adhered to positively charged slides using albumin, which served as both a modifier and an adhesive.
3- A series of ethanol solutions ranging from 70% to 100% should be prepared: Use distilled or deionized water to dilute 100% ethanol. You may reuse these solutions after storing them in the right containers.
4- Solution for Pretreatment with Heat Sodium EDTA: Bring a covered staining jar to a temperature of 98°C.
5- One part of 20x Wash Buffer TBS is diluted with nineteen parts deionized or distilled water to make 1x Wash Buffer TBS. When kept at 2-8°C, diluted 1x Wash Buffer TBS remains stable for one week.
6- For a stringency wash, bring 1x Wash Buffer TBS to 55°C in a covered staining jar.
7- Quick Fix ISH by ZytoFast Hybridization temperature and complete mixing are prerequisites for using the probe.

The data was analyzed using SPSS, version 25, and Excel was also used for the creation of graphical figures and charts.

Results:

Classification of all block samples:

Evaluation of EBV Signals according to intensity & patterns of replication between study groups:

When analyzing breast carcinoma cases using the IHC technique, the most common results for EBV intensity signals were weak intensity (16 cases, or 32% of the total), moderate intensity (14 cases, or 28%), and strong intensity (4 cases, or 8% of the total). In contrast, the control group had a high percentage of weak intensity (2 cases, or 6.7% of the total), moderate intensity (1 case, or 3.3% of the total), and strong intensity (0 cases, or 0% of the total) positive results. Table (1) shows that there were significant differences (P<0.05) across the study groups.

The results of the EBV EBER RNA signals by CISH technique in breast carcinoma were found in two groups based on replication patterns: mixed (episomal & integrated DNA) and punctate (integrated DNA). In the first group, 11 cases (22%) were detected, followed by 9 cases (18%) in a diffused pattern (episomal DNA). In the control group, there were 2 cases (6.7%) in punctate (integrated DNA), 1 case (3.3%) in diffused, and no cases (0%). When analysing the findings according to signals of intensities and patterns of replication, as indicated in table (2), there were significant differences (P<0.05) between the research groups

Table (1): Frequency distribution of positive EBV signal intensity between study groups by IHC

Study Groups	Total IHC +ve results	Positivity according to signal intensity			Total IHC -ve results
		weak	Moderate	strong	
Case / 50	**34 /50** (68 %)	**16** (32%)	**14** (28%)	**4** (8%)	**16 /50** (32 %)
Control / 30	**3 /30** (10%)	**2** (6.7%)	**1** (3.3%)	**0** (0%)	**27 /30** (90 %)
Total / 80	**37**	**18** 48.7%	**15** 40.5%	**4** 10.8%	**43**
		P-value:0.008, p<0.05, Significant			

Table (2): Frequency distribution of positive EBV (EBER) patterns of replication between study groups by CISH

Study Groups	Total CISH +ve results	Positivity according to signal pattern of replication			Total CISH -ve results
		Diffused	Punctate	Mixed	
Case / 50	**31 /50** (62 %)	**9** (18%)	**11** (22%)	**11** (22%)	**19 /50** (38 %)
Control / 30	**3 /30** (10%)	**1** (3.3%)	**2** (6.7%)	**0** (0%)	**27 /30** (90 %)
Total / 80	**34**	**10** 29.4%	**13** 38.2%	**11** 32.3%	**46**
		P-value:0.041, p<0.05, Significant			

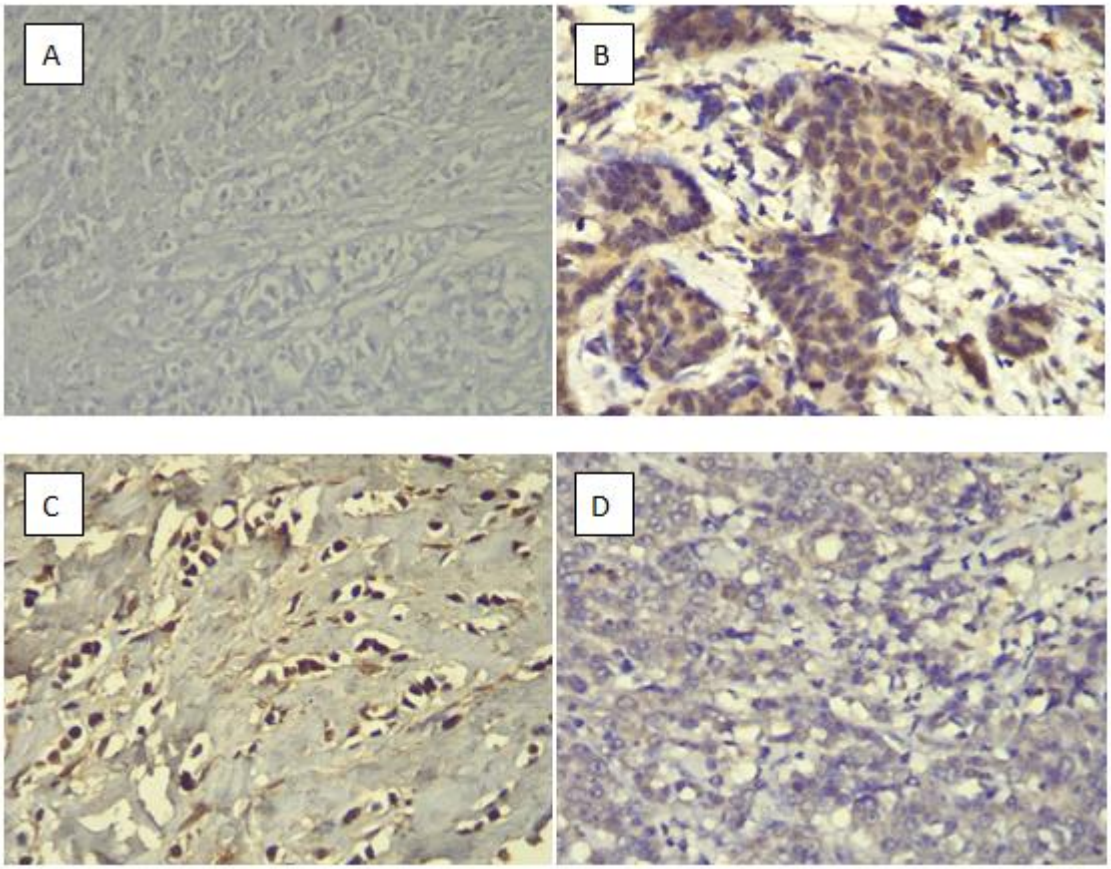

Figure (1): EBV EBNA-1 protein expression identified in IDC, cytoplasmic staining, A-Negative control, IHC, high power (x40)., B-Moderate intensity, IHC, high power (x40). C-strong intensity, IHC, high power (x40)., D-Weak intensity, IHC, high power (x40).

Discussion

When it comes to cancers affecting women, breast cancer ranks first globally [22,23]. Similarly, Iraq has a serious public health issue with breast cancer, which is also the most common disease in the country [24-26]. Even though the exact origin of breast cancer is still a mystery and is most likely polygenic, recent studies have linked EBV infection to the development of breast cancer [27]. It is worth mentioning that there is a lot of research suggesting a link between EBV and the risk of breast cancer. A statistically significant correlation was shown in one meta-analysis [27].

Table (1) shows the subdivision of the EBV positive pattern found by IHC in our investigation into various signal strengths. Weak, moderate, and strong positives were detected in 32%, 28%, and 8% of cases, respectively, due to the varying intensities of EBV staining. Among the 30 controls, no significant EBV staining was detected by IHC, but 2 (7%) of the positive findings showed mild staining intensities, and 1 (3.3%) showed moderate intensity. It seems that there is a high likelihood of EBV infection in patients, as we found a statistically significant difference (p-value < 0.05) between the two groups. However, we couldn't use a statistical test that looked for variations in the categories of signal intensities between the cases and the controls since our sample size was too small. The use of immune-histochemistry to detect EBV-associated proteins is a key component of studies that investigate the presence of EBV in breast cancer tissue [28]. identified mild, moderate, and severe EBV-LMP1 immunohistochemistry signal intensity [28, 29], indicated that a more aggressive tumor was much more likely to be discovered when EBV infections

were found inside breast cancer tissues, regardless of the signal strength. Nonetheless, immunohistochemistry (IHC) targeting EBV proteins such as LMP1 and EBNA1 indicates positive staining in a range of 0-51% across several studies [29-31]. The specific features of EBV infection inside the tumor microenvironment and the intrinsic limits of IHC are two of the many variables that contribute to this heterogeneity [32]. The researchers noted in their study that invasive breast cancer patients had moderate to high staining intensities when tested with IHC for EBV's EBNA-1 [29]. Using polymerase chain reaction (PCR), they found that EBV was present in 51% of the 100 samples of primary invasive breast cancer. Immunohistochemistry staining for EBNA-1, a hallmark of EBV infection, showed that only a small percentage (5-30%) of tumor epithelial cells displayed the antigen. When tumors tested negative for EBV by PCR, no staining for EBNA-1 was seen. So, in situations where EBV was present, the staining intensity was moderate to high, but in cases where EBV was not, it was nil. When compared to healthy individuals, EBV is more often discovered in a range of different malignant tumors with strong positive IHC signals, in contrast to breast cancer, which has a varied signal strength [33, 34]. As an example, nasopharyngeal carcinoma (NPC) tumor cells only contain latent EBV, which is highly detectable by immunohistochemistry (IHC) [34]. In a similar vein, immunohistochemistry (IHC) reveals EBV-positive in EBV-associated gastric cancer (EBVaGC) [33].

[35], observed varying degrees of EBV/LMP1 staining, with a range of negative (34.7% of cases), mild (29.3% of cases), moderate (30.7% of cases), and severe (5.3%); moreover, [36] found weak EBV/LMP1 staining in 22.5% of HNSCC patients. These results raise the intriguing prospect that EBV may be implicated in certain HNSCC tumors, although with variable expression patterns. Among EBV-positive breast tumors, the researchers found an ISH signal strength ranging from moderate to high, suggesting that the infection may cause paclitaxel resistance [37]. These results demonstrate that EBV may affect the effectiveness of therapy. Another research that looked for EBV in breast tumors employed two different EBNA-1 proteins [30]. They found that the proportion of EBNA-1-positive cells varied substantially between tumors, suggesting that the length of time clinical samples were fixed may have played a role. Unfortunately, the staining intensities were not specified.

In our research, we found that EBV was detected in 31% of patients (31/50) when CISH was run. In 18% (9/31) of cases, diffuse staining was seen, indicating a broad viral presence, as shown in table (2). Additionally, 22% (11/31) of the samples showed punctate staining and mixed positive. A much lower rate of EBV positive was seen in the control group (10%, 3/50), with a clear distribution pattern (diffuse: 3.3% and punctate: 6.7%), and no mixed pattern was observed. The results of the IHC were supported by statistical analysis ($p < 0.05$). There is evidence that the EBV profile in patients and controls is different. We were unable to use a statistical test of significance to precisely evaluate the changes in the positivity patterns for cases and controls, comparable to table (1), due to the small sample size. Various studies on the identification of EBV in breast cancer tissues have documented the punctate pattern of EBV identified by CISH. The presence of EBV-encoded RNA (EBER) in certain areas of breast cancer cells, which may be both dispersed and punctate, is related to this pattern [38], Using the ISH for EBER and PCR for EBNA-1 methods, they found that 18 out of 40 (45%) breast cancer tissues in the Egyptian research and 14 out of 50 (28%) in the Iraqi study had EBV. These samples tested positive for EBV in a punctate pattern. Another research [39] used nested PCR to find EBV DNA type 1 in 27.02 percent of Iranian patients' ductal breast cancer tissues. CISH verified EBV for EBER, and it was punctually positive [40]. In addition to outlining the EBV latency profile in breast cancer cases in Argentina. Their goal was to identify EBV genomic DNA and EBNA1 in the samples by using in situ

hybridization (ISH) with monoclonal antibodies. Researchers found that 31 percent of patients had a positive EBV test, with samples showing a punctate pattern of staining. According to their research, breast cancer tissues tested positive for EBER using CISH, and the resulting pattern was punctate. Epstein-Barr Virus (EBV) detection via EBER-CISH involves seeing viral RNA in cell nuclei, which allows for the identification of EBV in tumors [41]. Most tumor cells in EBV-positive cancers show strong and consistent nuclear positivity when stained with EBER-ISH, suggesting a high copy number of the EBV genome [41]. A lower viral load is indicated by weaker or localized staining, which is distinct from this pattern. Curiously, several EBV-associated cancers, including nasopharyngeal carcinoma, Burkitt's lymphoma, and Hodgkin's lymphoma, have been shown to have widespread EBER-CISH staining [41]. Thus, EBER-ISH helps detect latent EBV infection in EBV-positive malignant tumors due to its characteristic widespread and intense nuclear staining [41]. Uniform nuclear staining for EBV across most tumor cells characterizes the diffuse pattern. This trend suggests that the infection with integrated EBV is more widespread. The chromosomes of the host cell incorporate the viral genome [34]. The widespread pattern of EBV identification with ISH has been more often documented in breast carcinomas. The punctate pattern contrasts with this [42].

Multiple research projects have shown that the same tumor may exhibit both diffuse and punctate EBV signals. This points to a varied pattern of EBV infection [43]. The inconsistent pattern of EBV identification in breast cancer cases has not been well documented. In conclusion, our study is similar to the findings [38–40] in that both studies emphasize patterns of EBV-positive in breast cancer tissues. Using CISH for EBER and PCR for EBNA-1, [38] detected EBV in 45% of Egyptian and 28% of Iraqi breast cancer tissues, which is in line with our findings of a 22% punctate pattern (11/31). Our results are consistent with those of [39], which found punctate EBER ISH patterns and EBV DNA type 1 in 30% of Iranian ductal breast cancer samples. We found the same 31% punctate pattern as previously reported in [40], which also found the EBV latency profile in Argentine breast cancer. Our results are in agreement with those of other somewhat compatible studies, such as [43, 44], which defined the punctate pattern as a localized episomal EBV infection; however, these researchers did not have any information on breast malignancy [34, 42]. detailed a dispersed pattern that hinted at integrated EBV infection; this finding was also noted in the current research (18%, 9/31), but it was not their primary worry in relation to breast cancer. Other studies that find conflicting findings include [45], which found a diffuse pattern in breast carcinomas twice as common as the reported punctate results. Our mixed staining 22% (11/31) and punctate results contradict the diffuse and strong homogenous nuclear staining reported by EBER-ISH in [41]. The staining patterns addressed in this research vary from those in [46], which focused on EBV-specific treatments and significant genomic integration.

Conclusion: A total of 64.3% of the 70 blocks are devoted to invasive lobular carcinoma and invasive ductal cancer. The results also show that in the demographic group we looked at, there was an overexpression of the RNAs representing EBNA-1 and EBV EBER.

Author Contributions

The study design and the experiments were done by ASAAD FAKHIR WASHIL. In addition, all authors analyzed the data and wrote the manuscript.

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Ethics:

The study protocol was reviewed by the Human Ethics Committee of the College of Medicine, University of Misan, Iraq.

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Conflict of interest:

There is no conflict of interest.

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