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DETECTION OF EPSTEIN-BARR VIRUS (EBV) IN WOMEN WITH BREAST CANCER IN IRAQ USING IN-SITU HYBRIDIZATION AND IMMUNOHISTOCHEMICAL TECHNIQUES

DETECÇÃO DO VÍRUS EPSTEIN-BARR (EBV) EM MULHERES COM CÂNCER DE MAMA NO IRAQUE UTILIZANDO TÉCNICAS DE HIBRIDIZAÇÃO IN-SITU E IMUNOHISTOQUÍMICA

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ABSTRACT

Background: 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 cancer, nasopharyngeal carcinoma, and bladder cancer. It has been newly associated with breast cancer. Globally, breast cancer affects more women than any other type of cancer. In Iraq, the prevalence of breast cancer is comparable. **Aims**: The study examined Iraqi women diagnosed with invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) to detect Epstein-Barr Virus Nuclear Antigen-1 (EBNA-1) and encoded RNA (EBER). **Methods**: 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. **Results**: EBER RNA signals were found in 31 (62%). EBER RNA signals were seen in 3 (10%) control group participants. Significant differences ($P<0.04$) were seen in EBV EBER RNA-positive signals among study groups. Immunohistochemistry showed nuclear brown staining in 34 (68%) breast cancer patients. Control group: 3 (10%). Discussion: The research identified a statistically significant correlation between EBV positivity and breast cancer among Iraqi women, especially concerning invasive ductal carcinoma. The results corroborate previous reports of elevated EBV levels in malignant breast tissues relative to controls. Although detection approaches such as CISH and IHC provide complementary insights, additional studies are needed. **Conclusions**: The study concludes that EBNA-1 and EBV EBER RNA were overexpressed in our population group.

Keywords: *Breast cancer, EBV DNA, CISH, IHC*

RESUMO

Introdução: O vírus Epstein-Barr (EBV) foi recentemente identificado em câncer de mama humano globalmente, potencialmente contribuindo para o início e progressão desta malignidade, bem como câncer

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gástrico, carcinoma nasofaríngeo e câncer de bexiga. Ele foi recentemente associado ao câncer de mama. Globalmente, o câncer de mama afeta mais mulheres do que qualquer outro tipo de câncer. No Iraque, a prevalência do câncer de mama é comparável. **Objetivos**: O objetivo do estudo examinou mulheres iraquianas diagnosticadas com carcinoma ductal invasivo (IDC) e carcinoma lobular invasivo (ILC) para detectar o Antígeno Nuclear 1 do Vírus Epstein-Barr (EBNA-1) e RNA codificado (EBER). Métodos: Um total de 50 tecidos fixados em formalina e embebidos em parafina de amostras de biópsia de carcinoma ductal invasivo (IDC) (92%) e carcinoma lobular invasivo (ILC) (8%) constituíram o grupo de casos, enquanto 30 tecidos fixados em formalina e embebidos em parafina de tecido mamário não canceroso serviram como grupo controle. A presença da proteína do vírus Epstein-Barr (EBER) no tecido mamário foi avaliada usando métodos de imunohistoquímica (IHC) e hibridização in situ cromogênica (CISH). **Resultados**: Sinais de RNA EBER foram encontrados em 31 (62%). Sinais de RNA EBER foram observados em 3 (10%) participantes do grupo controle. Diferenças significativas (P<0,04) foram observadas nos sinais positivos de RNA EBER do EBV entre os grupos de estudo. A imunohistoquímica mostrou coloração nuclear marrom em 34 (68%) pacientes com câncer de mama. Grupo controle: 3 (10%). **Discussão**: A pesquisa identificou uma correlação estatisticamente significativa entre a positividade para EBV e o câncer de mama entre mulheres iraquianas, especialmente em relação ao carcinoma ductal invasivo. Os resultados corroboram relatos anteriores de níveis elevados de EBV em tecidos mamários malignos em relação aos controles. Embora abordagens de detecção como CISH e IHC forneçam insights complementares, estudos adicionais são necessários. **Conclusões**: O estudo conclui que EBNA-1 e RNA EBER do EBV foram superexpressos em nosso grupo populacional.

Palavras-chave: *Câncer de mama, DNA do EBV, CISH, IHC*

1. INTRODUCTION

Globally, breast cancer affects more women than any other type of cancer (DeSantis *et al*., 2018). The situation is quite similar in Iraq, where breast cancer is similarly seen as a significant public health issue and the most common type of cancer (Hussain and Lafta,2021; Al Alwan,2022).

New evidence is connecting EBV infection to breast carcinogenesis, even though the exact causation of breast cancer is still uncertain and probably polygenic (Jin *et al*., 2020). Significant evidence suggests a link between EBV and an increased risk of breast cancer, which is an important finding. A statistically significant correlation was shown in one meta-analysis that was carried out (Jin *et al*., 2020). Even more so, EBV was more common in breast cancer tissues than in normal control tissues. There is a robust statistical connection, as shown by additional meta-analyses (Hsu *et al*., 2024).

Herpes simplex virus type 4, is more often known as the Epstein-Barr virus (EBV). This particular gamma-herpes virus is unique to humans. Animals do not serve as reservoirs. In 1964, Denis Burkitt identified and described the virus from lymphoma samples; two scientists, Anthony Epstein and Yvonne Barr, were responsible for the virus's naming (Jawetz *et al*., 2010).

Up to 95% of adults between the ages of 35 and 40 have been infected with EBV, and most people will contract the virus at some point in their lives (National Health Service, 2010). Numerous human neoplasms have been linked to EBV, the most common of which are endemic Burkitt lymphomas and undifferentiated nasopharyngeal carcinomas (NPCs). Other tumor types that have shown viral DNA include tonsil, salivary gland, and thymus carcinomas, as well as a small number of cases of malignancies affecting the female genital tract (Kutok and Wang,2006; Eligio *et al*., 2010).

One possible role for EBV in cervical tumor pathogenesis has been suggested. A hitherto unrecognized link between EBV and cervical squamous cell cancer. Several autoimmune diseases, including dermatomyositis, rheumatoid arthritis, Sjögren's syndrome, multiple sclerosis, and systemic lupus erythematosus, have been linked to viral infections in recent years (Toussirot and Roudier, 2008; Villegas *et al*., 2010; Lünemann, 2012).

EBV is involved in several aspects of host cell carcinogenesis, including immune system cells (lymphocyte) transformation. It can convert lymphocytes in vitro and is, hence, the most potent oncogenic virus (Thorley *et al*., 2008). note that the specific mechanisms behind B-cell transition remain unclear. Infected B lymphocytes are able to enter the cell cycle, continue to proliferate indefinitely, and avoid cell death because the virus uses CD21 and HLA class II molecules on their surface to do so (Klein *et al*., 2007). Even though no virus is being generated in these dormant cells, the EBV genome is being retained as a multicopy episome.

The Immunohistochemistry technique brings about several advantages. In contrast to

approaches that merely detect EBV DNA, this approach enables the direct detection of EBV proteins in tumor cells, specifically EBNA-1, and thus provides stronger evidence of an active EBV infection (Khabaz, 2013; Joshi *et al*., 2009). Additionally, IHC's standardized techniques guarantee consistency of data across different research, and its extensive availability in pathology labs corroborates its efficacy (Shahi *et al*., 2022).

At the level of the tumor microenvironment, IHC can detect the presence of the virus in certain cell types and also determine which cell types the virus targets. Additionally, the method can identify EBV across the whole tissue area (Khabaz, 2013; Joshi *et al*., 2009).

However, there are some shared considerations that we need to make. The method's potential for producing false-positive results due to non-specific responses against cellular proteins reduces its specificity for identifying EBV infections (Shahi *et al*., 2022). Another major drawback is that PCR can identify the entire EBV genome, whereas IHC can only detect EBV-specific proteins (Shahi *et al*., 2022). The proportion of tumor cells that test positive for EBV among malignant cells might vary greatly, ranging from 5% to 50%. When the EBV expression level is low among cases, this diversity of variation could lead to an underestimate (Khabaz *et al*., 2017).

When looking for Epstein-Barr virus in breast tumor tissues, chromogenic in situ hybridization stands out as a superior technique compared to the fluorescence in situ hybridization (FISH) assay. A chromogenic detection system is the main mechanism by which CISH operates. Because of this method, the specialized and costly fluorescence microscopy is unnecessary, and the examiner may see the results with a regular light microscope.

According to several studies (Isola and Tanner,2004; Jensen,2014; Rosa *et al*., 2013; Hanna and Kwok, 2006), CISH is the most practical and economical method for doing these types of tests in a laboratory setting, making it a great substitute for the highly specialized and costly FISH technique. As a second point, CISH's chromogenic signal is far more stable than FISH's fluorescent signal. This allows the material to be stored permanently for further testing or analysis. Then, the examiner can simultaneously observe tissue structure and EBV detection using CISH. Visible in the tissue, the chromogenic signal adds to our understanding of the tissues' histopathological state (Rosa *et al*., 2013).

1.1. Aim of study

1- Immunohistochemistry and the chromogenic in situ hybridization technique will be used to identify EBV DNA in breast malignant tissues and compare the results with those from normal breast tissues in order to evaluate any potential function of EBV in the progression of breast cancer.

2- Researching the age distribution of Iraqi women diagnosed with breast cancer and the factors that may influence their prognosis in order to develop more effective treatments and preventative measures.

3- Look into the possibility that the virus has a role in the onset or advancement of breast cancer.

4- To give a cytodiagnostic gold standard for comparison other than histology.

2. MATERIALS AND METHODS

Utilizing immunohistochemistry and in situ hybridization methods, this study assessed the frequency of EBV human herpes virus in breast cancer cases among Iraqi women residing in the southern region of the country. The research utilized eighty breast tissue blocks preserved in formalin and embedded in paraffin. Of these, forty-five blocks included breast carcinomas for the case study, and thirty blocks contained non-malignant breast tissues for the control. The blocks with better fixation and processing were used in the case study.

2.1. Materials

2.2.1. A - Malignant group (case of study):

The number of instances of breast cancer included in this study is fifty (50). The demographic parameters and patient ages were documented on the case sheets. Their ages varied from twenty-five to seventy-three.

2.2.2. B - Non-malignant group (control of study):

A total of thirty breast tissues, all of which were determined to be free of malignancy by pathology and preserved in paraffin, served as the control group. Ages varied from twenty-four to seventy-two.

From January 2022 to December 2023, all specimens, whether malignant or non-malignant, were included in the patient blocks. Maysan Governorate's Al-Sadr General Hospital and Central Public Health Laboratories were consulted for the selection of malignant and non-malignant blocks. Hematoxylin and eosin (H&E) stained tissue blocks were used to diagnose these patients based on pathological data found in laboratory files. We gathered information about patients' ages, dates of birth, tumor sizes, grades, and histological examinations by reviewing their pathological reports and re-reading their clinical records. The room temperature was used to store all samples.

2.2. Methods

2.2.1. Sample preparation:

The research and control groups' paraffin-embedded tissue blocks were gathered. Each of the blocks that had paraffin embedded was used to make new portions in the following ways: On unspecial slides, four (4) µm-thick slices were cut. Certified pathologists reviewed newly manufactured hematoxylin and eosin (H&E) stained slides and validated the diagnosis of cancer types and grades according to criteria given by the World Health Organization (WHO). All relevant histological slides were re-examined.

Two further sections, each 4 µm thick, were cut on positively charged slides in order to carry out the in-situ hybridization procedure and the Immunohistochemistry (IHC) technique, which are used to detect the presence of EBV DNA.

2.2.2. Staining procedure of IHC

The immunohistochemical analysis was performed using the ZytoChem Plus HRP Kit detection system. Following a 10-minute wash with buffer solution, slides were treated with 3% H_2O_2 solution, then incubated with blocking solution (protein block, Reagent 1) for 5 minutes.

Primary antibodies at optimal dilution (or negative control reagent) were applied for 30-60 minutes, followed by three 2-minute washes with buffer solution. The biotinylated secondary mouse antibody (Reagent 2) was then applied for 30-60 minutes. After three additional 2-minute washes, streptavidin-HRP-conjugate (Reagent 3) was used for 10-15 minutes, followed by three final 2-minute washes.

Visualization was achieved using a DAB solution (5-15 minutes) with the reaction terminated by distilled water. Mounting options included aqueous mounting with AEC or permanent mounting with either DAB or AEC.

For analysis, two independent pathologists examined each slide using a LEICA DM750 light microscope (Germany). Cell counting was performed at 400× magnification across 10 randomly selected fields. Nuclear brown staining in tumor cells was considered positive for the target protein.

2.2.3. In situ hybridization

Using chromogenic in situ hybridization (CISH), the ZytoFast EBV Probe may detect human Epstein-Barr virus (EBV) EBER RNA in formalin-fixed, paraffin-embedded specimens. This probe is designed to be used in conjunction with the ZytoVision Bremerhaven/Germany HRP-DAB, which is part of the ZytoFast PLUS CISH Implementation Kit High Sensitivity. There is only one size of the ZytoFast EBV, which is made up of Digoxigenin-labeled oligonucleotides (~ 0.2 ng/µl) that target mRNA sequences encoding EBER-1 and EBER-2 regions.

- We followed the instructions on the packaging for the ZytoVision DNA probe hybridization/Detection system in situ kit to make sure all of our chemicals were ready to use.
- To create 20x Wash Buffer TBS (WB5), follow the steps outlined in the assay protocol.
- No additional reagents in the kit are required for use. There is no need to dilute, mix, or reconstitute.
- The oven was used twice to sterilize the glassware, each time for four hours at 100 ˚C. The pipette tips, distilled water, and Eppendorf tubes were autoclaved for 20 minutes at 121 ˚C to ensure sterilization.
- Tissues known to contain the target marker were used to generate the positive tissue slide, which served as the control. For EBV, they accounted for nasopharyngeal carcinoma.
- In situ hybridization runs were accompanied by a negative control. With the exception of the diluted probe, all reagents were added to the negative control.

Epstein-Barr virus (EBV) EBER RNA is used as a radioactive molecular marker in in situ hybridization. With this hybridization/detection system used correctly, it will produce A nuclear staining pattern that indicates that the target cells

have a favorable reaction to Epstein-Barr virus (EBV) EBER RNA. When detected by horseradish peroxidase (HRP) and diaminobenzidine (DAB) at the particular position of the hybridization probe in positive test tissue, hybridized digoxigenin-labeled oligonucleotides emerge as a brown pattern using the ZytoFast PLUS CISH Implementation Kits. Infected cell nuclei were the primary locations of the positive hybridization signals.

3. RESULTS AND DISCUSSION

3.1. Results

Figure 1 illustrates the histopathological features of invasive ductal carcinoma (IDC) across three distinct differentiation grades. Moreover, the results for the same tissue with different Magnifications (4x,, and 40x) are displayed in supplementary figures for all grades (1S, 2S, 3S). The study analyzed 50 breast cancer cases, revealing that grade 2 (moderately differentiated) carcinoma predominated at 52% (26 cases), followed by grade 3 (poorly differentiated) at 30% (15 cases) and grade 1 (well-differentiated) at 18% (9 cases).

The hematoxylin and eosin (H&E) stained sections (400× magnification) demonstrate the progressive morphological alterations associated with increasing histological grade:

Panel A (Grade 1 IDC): This well-differentiated carcinoma exhibits tumor cells arranged in cohesive clusters with well-formed tubular structures. The neoplastic cells display minimal nuclear pleomorphism with relatively uniform, round-to-oval nuclei and inconspicuous nucleoli. Mitotic figures are rare, and the intervening stroma appears abundant. These features reflect a tumor that largely retains the architectural organization of normal breast tissue, correlating with less aggressive biological behavior and a more favorable prognosis.

Panel B (Grade 2 IDC): This moderately differentiated carcinoma demonstrates increased cellularity with moderate nuclear pleomorphism. Tubule formation is significantly reduced compared to grade 1, with tumor cells forming less organized glandular structures. Nuclear size and shape show greater variability, with more prominent nucleoli and occasional mitotic figures. The reduced stromal component and increased cellular density indicate intermediate differentiation and biological aggressiveness.

Panel C (Grade 3 IDC): This poorly differentiated carcinoma is characterized by sheets and nests of pleomorphic tumor cells with minimal to absent tubule formation. Nuclear features include marked variation in size and shape, prominent nucleoli, and coarse chromatin. Mitotic activity is typically elevated, and areas of comedo-type necrosis may be present. These histological features correspond to aggressive biological behavior and poorer clinical outcomes.

The observed distribution of histological grades (18% Grade 1, 52% Grade 2, and 30% Grade 3) aligns with the typical pattern reported in the literature for invasive ductal carcinoma, with a predominance of moderately differentiated tumors. This distribution has significant prognostic implications, as histological grade serves as an independent predictive factor for survival and therapeutic response.

The Nottingham Histologic Score (modified Scarff-Bloom-Richardson grading system) used in this assessment evaluates three morphological features: tubule/glandular formation, nuclear pleomorphism, and mitotic count. This standardized grading system remains fundamental in determining the biological behavior of breast carcinomas and guiding therapeutic decision-making.

3.1.1. Associations between breast carcinoma types & grades

In this study, fifty cases of breast carcinoma were examined. The invasive ductal carcinoma (IDC) was categorized according to tumor grades as follows: 7 (14%) were grade 1, 25 (50%) were grade 2, and 14 (28%) were grade 3. As shown in Figure 2, the invasive lobular carcinoma (ILC) cases were classified as follows: 2 (4%) were grade 1, 1 (2% for grade 2), and 1 (2% for grade 3). There were no discernible variations ($P>0.05$) in the types and grades of breast cancer.

3.1.2. Distribution of age group patients with breast carcinoma according to histological types

According to Figure 3, the histopathological analysis of breast carcinoma cases (N=50) demonstrated distinct patterns in the distribution of histological subtypes across different age cohorts. Invasive ductal carcinoma (IDC) was the predominant histological variant, accounting for 92% (n = 46) of all cases, while invasive lobular carcinoma (ILC) represented 8% (n = 4) of the study population.

Among IDC cases, a bimodal age distribution was observed with the highest incidence in the 40-49 year age group (n=14, 30.4% of IDC cases), followed by patients aged 60 years and above (n=13, 28.3%). The 50-59 year age group accounted for 12 cases (26.1%), while patients younger than 39 years represented the smallest proportion (n = 7, 15.2%). This distribution pattern aligns with epidemiological data reported in similar regional studies, though the relatively high proportion of patients under 50 years (45.6% of IDC cases) may reflect the younger age structure of the population or potential genetic and environmental factors specific to the study region.

The limited number of ILC cases (n=4) exhibited a notable concentration in the 40-49 year age group (n=3, 75% of ILC cases), with a single case reported in patients under 39 years (25%). Notably, no ILC cases were documented in patients aged 50 years or older. While this distribution pattern for ILC differs from typical Western data, where ILC is more frequently observed in older patients, the small sample size precludes definitive conclusions regarding age-specific ILC prevalence in this population.

Statistical analysis revealed no significant differences between histological subtypes across age groups ($p < 0.038$), suggesting that age alone may not be a determinative factor for developing specific histological variants of breast carcinoma in this cohort. However, the distinctive age distribution patterns observed warrant further investigation with larger sample sizes to elucidate potential age-related biological mechanisms underlying different histological presentations of breast carcinoma.

These findings may have implications for screening strategies and clinical management, particularly considering the substantial proportion of breast cancer cases occurring in younger women in this population compared to Western cohorts.

3.1.3. Distribution of age group patients with breast carcinoma according to histological grades

According to Figure 4, the analysis of breast carcinoma cases (N=50) reveals a distinct distribution pattern of histological grades across different age cohorts. Grade 2 (moderately differentiated) carcinoma emerged as the predominant histological classification, comprising 52% (n = 26) of all cases, followed by Grade 3 (poorly differentiated) at 30% (n = 15) and Grade 1 (well-differentiated) at 18% (n = 9).

The distribution of Grade 2 carcinoma demonstrated a notable concentration in the 40-49 year age group, with 11 cases (42.3% of all Grade 2 cases) occurring in this cohort. The remaining Grade 2 cases were distributed equally between the 50-59 and ≥ 60-year age groups (6 cases each, representing 23.1% of Grade 2 cases per group), while only 3 cases (11.5%) were observed in patients younger than 39 years.

Grade 3 (poorly differentiated) carcinoma exhibited a more uniform distribution across younger and middle-aged cohorts, with 4 cases each in the <39 and 40-49 year groups (26.7% of Grade 3 cases per group) and 5 cases (33.3%) in the 50-59 year group. Significantly, only 2 cases (13.3%) were identified in patients aged ≥60 years, suggesting a possible inverse relationship between age and tumor aggressiveness.

Grade 1 (well-differentiated) carcinoma exhibited a distinctive age-related pattern, with a marked predominance in the ≥60-year age group (5 cases, 55.6% of all Grade 1 cases). The remaining Grade 1 cases were distributed sparsely across other age groups, with 2 cases (22.2%) in the 40-49 year cohort and only 1 case (11.1%) in each of the <39 and 50-59 year groups.

Statistical analysis revealed no significant differences in the distribution of histological grades across age groups ($p = 0.057$), although the p-value approached the threshold for statistical significance. This near-significant trend suggests potential age-related biological differences in breast carcinogenesis that may be confirmed statistically with a larger sample size.

The observed distribution patterns align with established literature, suggesting that well-differentiated tumors tend to occur more frequently in older patients, while poorly differentiated, more aggressive tumors are relatively more common in younger age groups. These findings may have implications for age-specific screening protocols and therapeutic approaches, particularly in populations with demographic and genetic characteristics similar to those of our study cohort.

3.1.4. Evaluation of EBV signals between study groups

According to Table 1, Epstein-Barr virus (EBV) detection rates in breast tissue samples showed marked differences between case and

control groups, as determined by both chromogenic in situ hybridization (CISH) and immunohistochemistry (IHC) techniques. The study encompassed 50 breast carcinoma cases and 30 control samples from non-malignant breast tissue.

The CISH method, which detects EBV-encoded RNA (EBER), revealed positive signals in 31 of 50 breast carcinoma cases (62%), characterized by brown discoloration at the site of complementary sequences in the nucleus of infected cells. In contrast, only 3 of 30 control samples (10%) exhibited positive EBER signals. The difference in EBV detection between case and control groups using CISH was statistically significant ($p<0.004$), suggesting a strong association between EBV presence and breast malignancy.

Similarly, IHC analysis demonstrated positive EBV signals in 34 of 50 breast carcinoma cases (68%), evidenced by nuclear brown staining of tumor cells using horseradish peroxidase (HRP) detection with DAB chromogen. Among the control group, only 3 of 30 samples (10%) showed positive IHC signals. This difference was also statistically significant ($p < 0.003$), further corroborating the association between EBV and breast carcinoma.

The data reveal a high level of concordance between CISH and IHC methods, with marginally higher detection rates using IHC (68%) compared to CISH (62%) in breast carcinoma cases. This slight difference could be attributed to the distinct molecular targets of each technique—CISH detects viral RNA while IHC identifies viral proteins, potentially capturing different stages of viral activity within tumor cells.

The highly significant p-values ($p < 0.004$ for CISH and $p < 0.003$ for IHC) strongly support the hypothesis that EBV infection is associated with breast carcinogenesis. The substantially higher detection rates in malignant tissue (62-68%) compared to non-malignant control tissue (10%) suggest that EBV may play a role in breast cancer pathogenesis rather than representing incidental colonization.

These findings align with emerging evidence implicating EBV in breast carcinogenesis, though the precise mechanisms through which the virus may contribute to malignant transformation remain to be fully elucidated. The dual-method approach employed in this study provides robust technical validation for the observed association between EBV and breast carcinoma.

3.2. Discussions

Globally, breast cancer affects more women than any other type of cancer (DeSantis *et al*., 2018). The situation is similar in Iraq, where breast cancer represents a significant public health issue and remains the most common malignancy (Hussain and Lafta,2021; Al Alwan,2022).

Emerging evidence connects Epstein-Barr virus (EBV) infection to breast carcinogenesis, although the exact etiology of breast cancer remains uncertain and is likely polygenic (Jin *et al*., 2020). Several studies suggest a significant link between EBV and increased breast cancer risk. A meta-analysis demonstrated a statistically significant correlation between EBV infection and breast cancer development (Jin *et al*., 2020). Furthermore, EBV prevalence was higher in breast cancer tissues compared to normal control tissues, with additional meta-analyses confirming this robust statistical association (Hsu *et al*., 2024).

However, the literature presents some discrepancies. Certain studies failed to establish a connection between EBV and the clinicopathological features of breast cancer tumors (Salih *et al*., 2022). Some researchers suggest that negative EBV findings in breast cancer tissues by immunohistochemistry (IHC) might result from limitations in detection techniques rather than the actual absence of the virus (Deshpande *et al*., 2002).

These conflicting results highlight the necessity for accurate EBV detection in breast cancer patients. The methodology employed in our study is particularly significant as it combines polymerase chain reaction (PCR) and chromogenic in situ hybridization (CISH) with immunohistochemistry (De *et al*., 2022; Gutjahr *et al*., 2023). EBER-CISH (EBV-encoded RNA chromogenic in situ hybridization) is considered the gold standard for identifying EBV infection in cancerous tissues due to its precise localization capabilities and high sensitivity (Luk *et al*., 2019).

This case-control study, therefore, aims to investigate analytical indicators of Epstein-Barr virus infection in breast tumors, contributing to the ongoing research exploring the potential association between EBV and breast carcinogenesis.

3.2.1. Histological types & grades of breast carcinoma cases

In this study, invasive ductal carcinoma (IDC) accounted for 92% of cases (46/50), while invasive lobular carcinoma (ILC) represented 8% (4/50). Analysis of histological grades revealed that grade 2 tumors predominated in 52% of cases, followed by grade 3 (30%) and grade 1 (18%), as illustrated in Figures 4-2 and 4-3.

Among IDC cases, the distribution by grade was: grade 1 in 7 patients (14%), grade 2 in 25 patients (50%), and grade 3 in 14 patients (28%). ILC was relatively uncommon, with only 4 cases: 2 cases of grade 1 (4%), 1 case of grade 2 (2%), and 1 case of grade 3 (2%), as shown in Figure 4-5. Statistical analysis revealed no significant difference between grades and histological subtypes ($p > 0.05$).

Our findings align with current literature indicating that IDC is the most common breast cancer subtype, typically presenting as grade 2, followed by grade 3 and grade 1 [27,28]. Previous studies report that IDC accounts for 75-80% of breast cancer cases, while ILC represents 10-15% (Howlader *et al*., 2020; Weigelt *et al*., 2010). The distribution pattern of IDC by tumor grade in our study confirms the pattern initially described by Elston and Ellis in 1991, with a predominance of grade 2 followed by grades 3 and 1.

Regarding ILC, the literature indicates that most tumors are of lower grade, with 60-80% classified as grade 1 or 2 (Li *et al*., 2005). Comparative studies between ILC and IDC have consistently shown that ILC generally exhibits a lower grade distribution. Denkert et al. characterized ILC as a "luminal A breast cancer prototype," typically presenting as a low-grade malignancy. However, Boughey et al. (2016) (Boughey and Nguyen, 2016) documented higher-grade HER2-positive ILC associated with more aggressive disease progression, while another study identified higher tumor grades in a specific ILC subtype compared to IDC (Mittendorf *et al*., 2014).

The predominance of grade 2 IDC in our cohort reflects the typical pattern described in the literature, with grade 1 being less frequent and grade 3 often observed in younger patients or those with more advanced diseases (Van *et al*., 2022). While both IDC and ILC can be classified as grade 3, the invasive nature and higher prevalence of IDC increase the likelihood of a grade 3 diagnosis in this subtype (Haagensen *et al*., 1978). Multiple studies support this observation, noting that higher-grade IDC constitutes a significant proportion of breast cancer cases (Rakha *et al*., 2010).

Our study's findings regarding the distribution of histological grades in IDC and the lower incidence of ILC are consistent with previous breast carcinoma research (Van *et al*., 2022; Jaroensri *et al*., 2022). The lower-than-expected prevalence of ILC in our cohort aligns with findings from other studies examining this less common breast cancer subtype.

3.2.2. Interpretations of the results of age group patients with breast carcinoma according to histological types

Our study revealed that invasive ductal carcinoma (IDC) was the predominant histological subtype, accounting for 92% ($n = 46$) of all cases. Among IDC patients, the 40-49 age group was most affected, with 14 cases, followed by the 60+ age group, with 13 cases. Invasive lobular carcinoma (ILC) accounted for only 8% ($n=4$) of cases, with the majority occurring in the 40-49 age bracket, as illustrated in Figure 4-8. Statistical analysis revealed no significant differences in outcomes between age groups or breast cancer subtypes ($p > 0.038$).

The incidence of invasive ductal carcinoma demonstrates considerable variation across different populations and geographical regions. A Nigerian study of 81 cases reported a mean diagnosis age of 45.06 years, with women aged 30-39 comprising approximately 38.3% of subjects (Ebughe *et al*. 2013). In contrast, a larger cohort study involving 279 breast cancer patients reported a wider age range with a mean age of 57 years (Tbeileh *et al*., 2024).

Regional disparities in IDC diagnosis age are notable. In Saudi Arabia, the median detection age is 54 years (mean, 55.68 years), with 61.7% of patients diagnosed before the age of 50 (Alabdulkarim *et al*., 2018). By comparison, the average diagnosis age in the United States and United Kingdom is 62 years, with a disproportionately high incidence among women aged 70 and older (DeSantis *et al*., 2018; Asiri *et al*., 2020). These variations highlight the influence of cultural, environmental, and geographical factors on IDC epidemiology, underscoring the need for early detection and sex-specific screening programs, particularly in regions where IDC prevalence is higher among younger women (Ebughe *et al*. 2013).

Invasive lobular carcinoma (ILC) typically

affects older women, though exceptions exist. A Nigerian study examining breast cancer cases (including ILC) reported an age range of 21-79 years, with a mean diagnosis age of 42.0 years (Ibrahim *et al*., 2015). Another study found a stronger correlation between mammographic density and ER-negative breast cancer (including ILC) in younger women (<55 years) compared to older women (Bertrand *et al*., 2013).

A comprehensive meta-analysis of 40 studies involving 87,303 patients reported a mean age of 54.9 years for ILC compared to 50.9 years for IDC (Jung *et al*., 2010). Further research indicates that patients diagnosed at or below this age group demonstrate lower overall survival rates compared to those in middle-aged categories (Liu *et al*., 2016). The increased prevalence of ILC among women over 50 years warrants additional investigation to inform appropriate preventive measures (Yang *et al*., 2020).

The age distribution patterns observed in our study and the reviewed literature suggest the need for age-appropriate screening strategies that account for the varying presentation of breast cancer subtypes. The younger presentation of IDC in certain populations underscores the importance of early detection programs, while the typically later onset of ILC highlights the continued need for screening in older age groups.

3.2.3. Interpretations of the results of age group patients with breast carcinoma according to histological grades

Another objective of our study was to look at the age distribution of breast cancer patients by histological grade. The subtype of breast cancer most commonly found in women aged 40–49 was grade 2, which accounted for 26 instances (52%). The age group most impacted was those between 50 and 59 years old, accounting for 30% of the total cases (15 diagnoses). Figure (4–9) shows that 9 out of the 100 participants (18%) had grade 1 carcinoma, with the majority of these cases being older adults (>60 years). A statistical analysis revealed no correlation ($p = 0.057$) between age group and breast cancer grade. The current study's findings support those of Nixon et al. (1994), which indicate that people aged 40 to 49 years old had a higher rate of grade 2 tumors, while those aged 50 to 59 years old had a higher rate of grade 3 tumors. On the other hand, it argues that age does not significantly impact cancer grade outcomes, which goes against our assertion that there is no robust correlation between the two. At the same time, we found an increased incidence of grade 3 tumors in older age groups (Alabdulkarim *et al*., 2018). However, showing the age and grade as having a complicated connection contradicts our conclusion that there is no major association. This study lends credence to the idea that age and cancer grade should not be considered together when making decisions, likewise suggests that there is no positive correlation between the two variables, but rather that age and breast cancer outcomes are correlated in a U-shaped fashion (Xie *et al*., 2023). Grade 2 tumors in younger girls and grade 3 tumors in older females are similar (Koshariya *et al*., 2022). Regarding the prevalence of grade 3 tumors in the 50-59 year old age group (Chen *et al*., 2016). Potential association between the higher-grade malignancies in senior females and similar data regarding the occurrence of grade 2 tumors in young females (Feizi *et al*., 2023). Despite our claims to the contrary, we found no correlation between age and cancer grade (Kroman *et al*., 2000). Aggressive tumor features and a poor prognosis in patients can be independently correlated with age, according to them. Contrarily, our earlier findings are somewhat contradicted, which state that younger patients exhibit more aggressive tumor subtypes and poorer overall survival. Our results show that older patients had a higher proportion of grade 3 tumors; however, this finding is expanded upon by suggesting that cultural and demographic variables may moderate the association between age, tumor grade, and survival (Alabdulkarim *et al*., 2018). Lastly, cast doubt on our results about the frequency of grade 3 tumors in older women, highlighting the fact that younger age is better associated with a worse prognosis and greater tumor grade (Cai *et al*., 2020).

3.2.4. Interpretation of EBV positivity among breast cancer cases and controls

As shown in Table 1 of our investigation, we compared 50 samples of breast cancer malignancy with non-malignant control tissues to determine the prevalence of EBV positivity by immunohistochemistry and chromogenic in situ hybridization. In a study involving 50 cases, 68% of the patients were found to have EBV antibodies detected by IHC and 62% by CISH. In comparison, 10% of the 30 control tissue samples tested positive for EBV antibodies by IHC or CISH. The statistical test findings for both approaches were significant, with a P-value less than 0.004.

There is a robust direct correlation between the EBV positivity status and the existence or absence of breast cancer, as shown by the statistically significant test findings. Although the precise percentages of EBV positivity differ, our study's results are consistent with others that have reached similar conclusions. Our results were consistent with those of Hussein (2013), who also found a statistically significant correlation between EBV-positive patients and controls. Also, a greater percentage of breast cancer patients were EBV-positive compared to healthy controls, suggesting a strong correlation between EBV infection and breast cancer (Mezher *et al*., 2013). A statistically significant correlation was found between IDC cases and EBV positivity, with a larger proportion of tumors displaying this status (Abd *et al*., 2021).

Research on EBV in Egyptian and Iraqi females with primary invasive breast cancer (PIBC) has shown promising results. In their study, they found that EBV was more prevalent in PIBC patients than in the control group (Zekri *et al*., 2012). Specifically, 45% of Egyptians and 28% of Iraqis tested positive for EBV, while 0% of the control group tested negative ($p < 0.05$). The distribution of many viruses, including EBV, in breast cancer tissues of Egyptian patients was examined in a study by Metwally et al. (2021). In contrast to normal breast tissue, 49% of breast cancer cases tested positive for EBV. The link between EBV positive and clinical outcomes of breast cancer patients was not, however, shown in this investigation.

Although the precise percentages of EBV positivity differ, our study's results are consistent with those of others that have reached similar conclusions and discovered a statistically significant correlation between EBV-positive patients and controls (Hussein, 2013). Also, a greater percentage of breast cancer patients were EBV-positive compared to healthy controls, suggesting a strong correlation between EBV infection and breast cancer (Mezher *et al*., 2013). A statistically significant correlation was found between IDC cases and EBV positivity, with a larger proportion of tumors displaying this status, according to research (Abd *et al*., 2021).

Research on EBV in Egyptian and Iraqi females with primary invasive breast cancer (PIBC) has shown promising results. EBV was more prevalent in PIBC patients than in the control group. Specifically, 45% of Egyptians and 28% of Iraqis tested positive for EBV, while 0% of the control group tested negative ($p < 0.05$) [55]. The distribution of many viruses, including EBV, in breast cancer tissues of Egyptian patients was examined [56]. In contrast to normal breast tissue, 49% of breast cancer cases tested positive for EBV. The link between EBV positive and clinical outcomes of breast cancer patients was not, however, shown in this investigation.

4. CONCLUSIONS

Recent data indicate that the Epstein-Barr virus may be involved in the development of breast cancer, particularly in aggressive forms of the disease. Our study revealed significantly higher EBV expression in breast cancer tissues compared to control specimens using both IHC and CISH methodologies, consistent with previous research demonstrating increased prevalence of EBV in malignant breast tissues. The results of the present study clearly establish that EBV was more frequently expressed in breast cancer tissues than in the control group, regardless of whether IHC or CISH evaluation methods were employed.

Both immunohistochemistry (IHC) and chromogenic in situ hybridization (CISH) are valuable tools for detecting EBV in breast cancer, although each presents certain limitations. Their combined application offers enhanced diagnostic utility and more reliable results. CISH analysis demonstrated notable variations in signal intensity and distribution patterns, including diffuse, punctate, and mixed staining. Similarly, EBV staining intensity in breast cancer tissues ranged from weak to moderate to strong, with considerable heterogeneity between samples. It appears that the role of the EBV signal intensity in breast cancer is still not clearly defined and requires further investigation.

Age represents a confirmed risk factor for breast cancer, with increased incidence in middle-aged women; however, the role of age in modulating the EBV-breast cancer relationship remains undefined. Evidence suggests that EBV positivity may correlate with more aggressive tumor characteristics, including larger tumor size, higher histological grade, hormone receptor negativity, and poorer prognosis. However, contradictory findings in the literature regarding associations between EBV and specific clinicopathological parameters warrant further investigation to establish definitive correlations.

The detection of EBV DNA in healthy control tissues could be attributed to subclinical infection, B-cell carriage, or technical factors, highlighting the importance of distinguishing

between persistent infection and active viral participation in carcinogenesis. This differentiation is critical for understanding the true role of EBV in breast cancer pathogenesis. The precise mechanisms through which EBV may contribute to breast carcinogenesis remain inadequately defined, necessitating additional research to elucidate potential viral-host interactions that could serve as therapeutic targets. Further investigation into the contradictions in current literature regarding EBV's relationship with clinicopathological characteristics would significantly advance our understanding of this virus's role in breast cancer development and progression.

5. DECLARATIONS

5.1. Study Limitations

The current study presents limitations that should be acknowledged, including a relatively modest sample size and recruitment from a single geographic region, which may restrict the generalizability of our findings to broader populations with different demographic and environmental characteristics.

5.2. Acknowledgements

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5.3. Funding source

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5.4. Competing Interests

The authors hereby declare that they have no competing financial, professional, or personal interests that could have influenced the design, execution, analysis, interpretation, or reporting of this research. All authors confirm that they are completely independent from any commercial entities or funding sources that may have a potential interest in the outcomes of this study.

5.6. Use of AI

The authors declare that artificial intelligence tools were utilized to assist in the translation and linguistic enhancement of the manuscript, which was originally written in Arabic. AI was employed exclusively to enhance the grammatical quality, clarity, and fluency of the English text, without altering the scientific content, presented data, or interpretations of the results. All statistical analyses, conclusions, and intellectual contributions remain the sole responsibility of the authors. The authors thoroughly reviewed the final manuscript to ensure the accuracy and integrity of all scientific content.

6. HUMAN AND ANIMAL-RELATED STUDIES

6.1. Ethical Approval

This study was conducted in accordance with the ethical standards outlined in the Declaration of Helsinki and was approved by the Human Ethics Committee of the College of Medicine, University of Misan, Iraq, under approval number MHD2025. Formalin-fixed, paraffin-embedded tissue samples were obtained from the pathology archives with appropriate anonymization to ensure patient confidentiality. Given the retrospective nature of the study and the use of archival specimens, the ethics committee waived informed consent.

6.2. Informed Consent

All individuals provided written informed consent before participating in this study. The University of Misan College of Medicine Ethics Committee approved the consent process MHD2025). The permission form clearly stated the study's goals, procedures, risks, benefits, data confidentiality, and freedom to withdraw without penalty. Participants were informed that scientific publications and conference presentations would utilize anonymized data and research conclusions. Personal identifiers were never to be shared. Before obtaining written agreement from low-literate individuals, the study methods and consent information were thoroughly conveyed. All signed consent forms are securely stored at our institution and can be requested by editorial boards under strict confidentiality guidelines.

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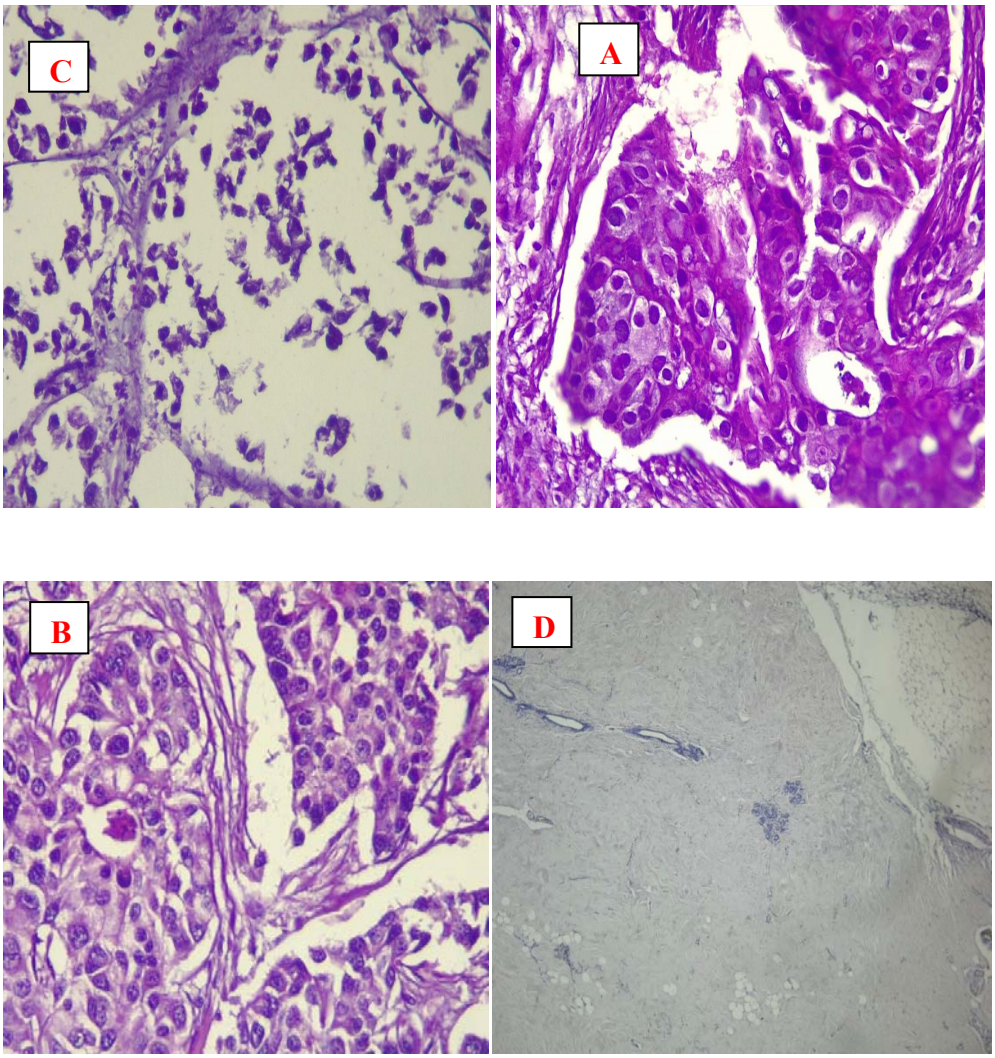

Figure (1): *Microscopic appearance of histological types of sections from tissue with invasive ductal carcinoma (IDC), stained with Hematoxylin & Eosin: A): Grade 1 invasive ductal carcinoma (X400) B) Grade 2 invasive ductal carcinoma (X400). C) Grade 3 invasive ductal carcinoma. D) Normal breast tissue.*

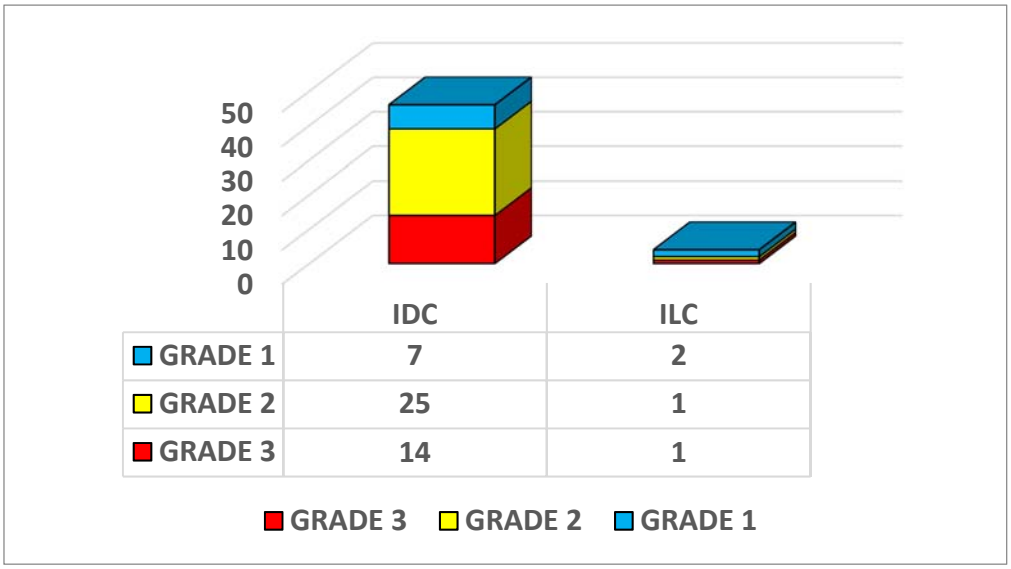

Figure 2: *Frequency Distribution of Histological Breast Carcinoma Types According to Grades*

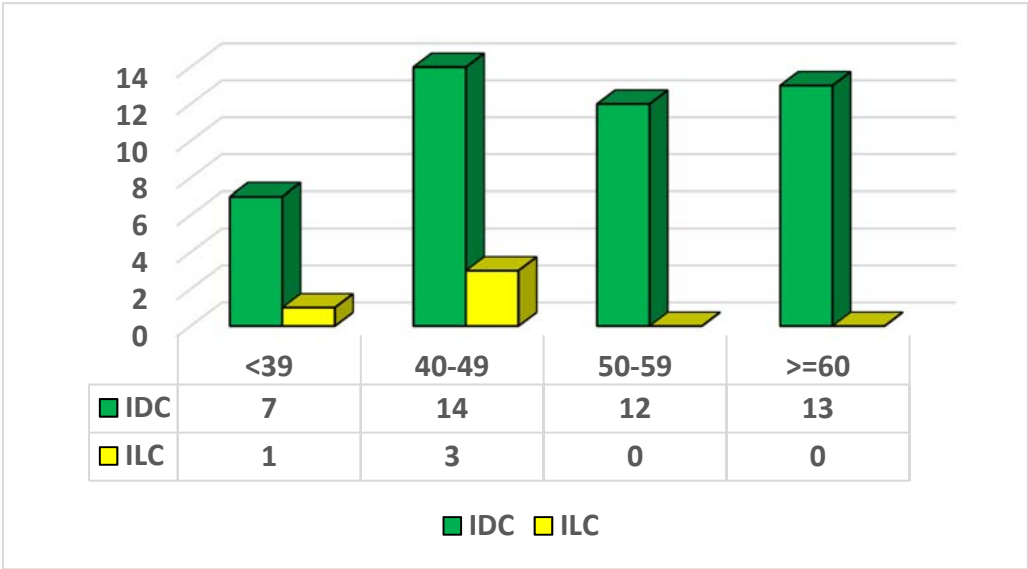

Figure (3): *Age Distribution According to Histological Types of Breast Carcinoma*

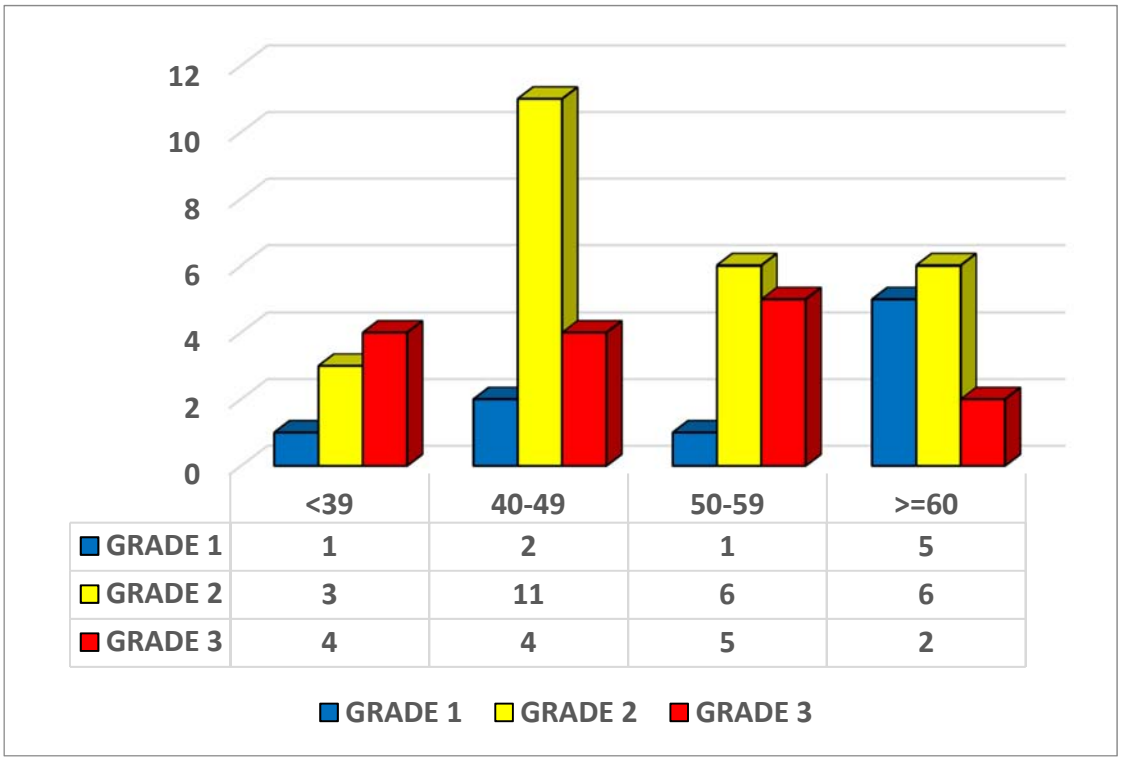

Figure (4): Age Distribution According to Histological Grades of Breast Cancer

Table 1: Total CISH and IHC results of EBV signal in the study group

Table 1: Total CISH and IHC results of EBV signal in the study group					
Study group	Total	EBV CISH results		EBV IHC results	
		+ve	-ve	+ve	-ve
Case	50	31(62%)	19(38%)	34(68%)	16(32%)
Control	30	3(10%)	27(90%)	3(10%)	27(90%)
Total	80	34	46	37	43
P-value between group	P<0.004			P<0.003	

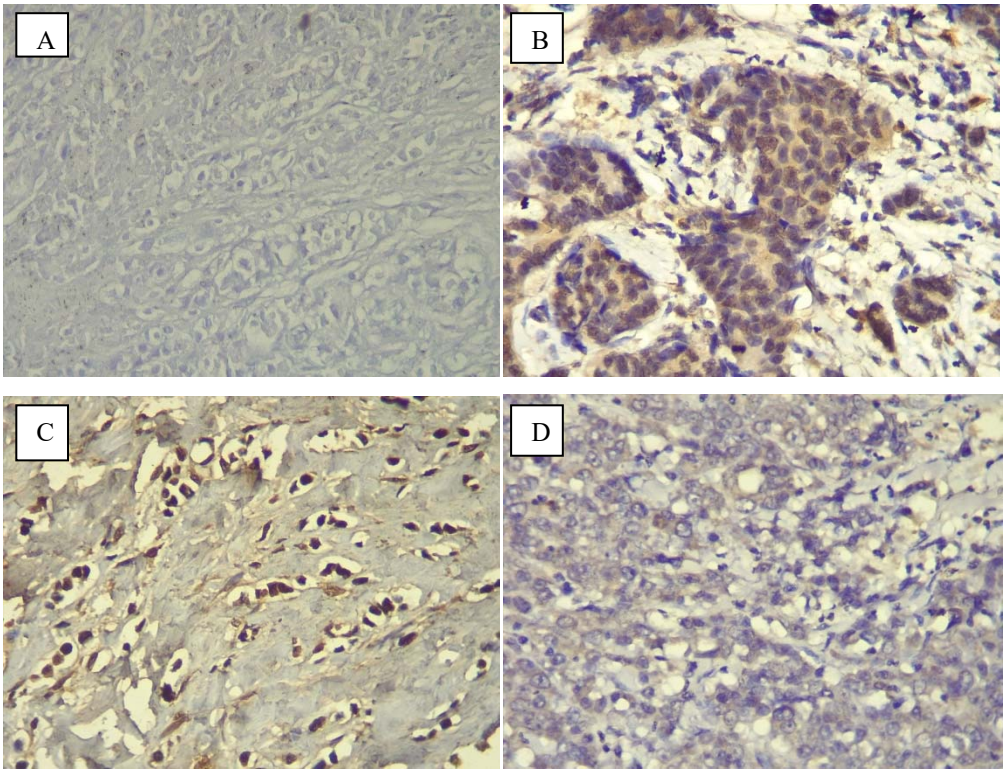

Figure (4-10): EBV EBNA-1 protein expression identified in IDC, cytoplasmic staining, A-Negative control, IHC, high power (x40). , B-Moderate intensity, IHC, high power (x40). , C-strong intensity, IHC, high power (x40). , D-Weak intensity, IHC, high power (x40).

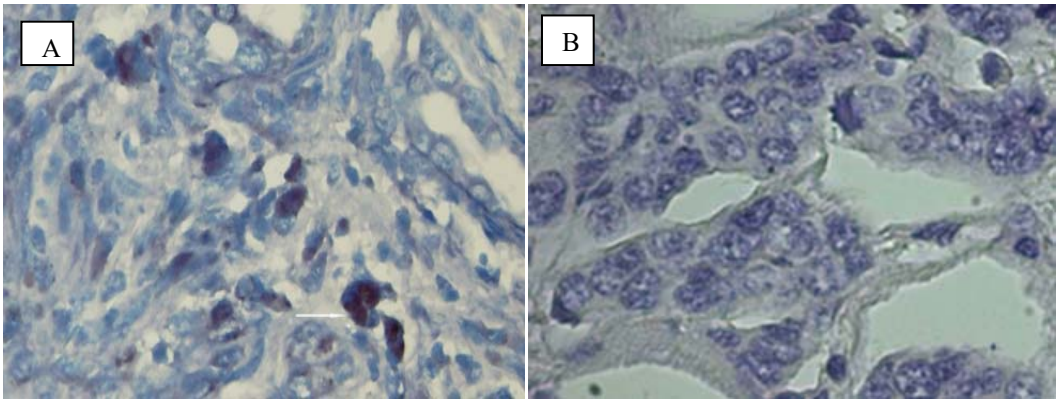

Figure (4-11): In situ hybridization staining of EBER in breast cancer section stained by DAB chromogen and counterstained with eosin (Magnification power, 400), A- EBER negative expression. (Mahjoub *et al*.,2008) B- EBER positive expression.