Prevalence of Epstein-Barr Virus and Human Cytomegalovirus Among Women with Breast Cancer in Basrah, Iraq

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Abstract
Breast cancer is the leading cause of death in women around the world and the most prevalent type of female cancer in Iraq. Accumulating evidence from the past ten years suggests that cytomegalovirus (HCMV) and the Epstein-Barr virus (EBV) are linked to various human cancers; including breast cancer. The present study aimed to detect the prevalence of EBV, HCMV, and the EBV genotype in formalin-fixed paraffin-embedded blocks (FFPE) of women with breast cancer and determine whether EBV and HCMV are associated with breast cancer. 45 Formalin-fixed and paraffin-embedded tissue blocks from 33 breast cancer and 12 benign breast tumors (control group) were collected from the pathology laboratories at Al – Fayhaa Teaching Hospital and Al–Sadder Teaching Hospital in Basrah province of Iraq which were used in this investigation. Using the UL55 gene, the PCR method detected HCMV DNA in 31/33 (93.9%) of breast cancer and 11/12 (91.7%) of benign breast tumors. EBV DNA was detected in 19/33 (57.6%)while among 12 benign breast tumors control group was EBV DNA positive 6 (50%).type1 EBV is the predominant type in these samples. The presence of both viruses in the same sample was found in (51.1 %) of the cases in this study. This study found that breast cancer with (IDC) and grade III had the highest percentage of both HCMV and EBV infection. In conclusion, the current findings indicate that EBV and/or HCMV may be associated with breast cancer.
Keywords: Breast cancer, EBV, HCMV, UL55 ,EBNA1

1. Introduction

Globally and in Basrah, Iraq, Breast cancer (BC) is a highly frequent disease. It is the most often diagnosed cancer in women and the main cause of cancer-related mortality (1–3). Compared to women in the West, Iraqi women experience breast cancer at least ten years earlier and in a more advanced state (4). The earliest age at first menstruation, nulliparity, hormone replacement treatment, ionizing radiation, alcohol, and obesity is the most prevalent risk factors for breast cancer (BC). However, particular inherited genetic abnormalities, mostly in the BRCA1 and BRCA2 genes, were responsible for 5- 10% of the cases (5). In substantial research, approximately 15% to 20% of malignancies are linked to virus infection `oncoviruses, and the fraction of each cancer type attributed to a viral infection varies widtablely (6). There are several risk factors linked to the development and progression of breast cancer, including a probable viral etiology (7,8). Oncogenic viral infections, such as those caused by the Epstein–Barr virus (9,10), the Human Papillomavirus (11), the Human Mammary tumor virus (12), and the Human Cytomegalovirus (13–15).
Epstein-Barr virus (EBV) or Human Herpes virus 4 (HHV4) belongs to the γ-herpes virus family (16). EBV is a group I carcinogen or an agent that certainly causes neoplasia in humans, according to the International Agency for Research on Cancer IARC (17,18). It was the first oncogenic virus discovered, Several diseases and malignancies have been linked to Epstein-Barr virus (EBV) and it causes lymphatic and epithelial malignancies (19). Some previous study has shown that approximately 95% of the world's population are infected, usually in childhood or early adolescence, with different manifestations and has an antibody response to the virus (20). The first link between EBV and breast cancer was revealed in 1995 (21). Relations between EBV and BC are debapic (22–24). Several investigations have shown that EBV infection increases malignant breast epithelial cell transformation and influences patient prognosis (25,26). 'Epstein–Barr virus infection predisposes breast epithelial cells to malignant transformation through activation of HER2/HER3 signaling cascades HER2 and HER3 are two of the cellular oncogenes known to be involved in human breast cancer development and associated with a relatively poor prognosis (25).
Increasing evidence in the last 10 years suggests that human cytomegalovirus (HCMV) is associated with several human malignancies, including malignant glioma, colorectal carcinoma, prostate cancer, and skin cancer, and that HCMV gene products can modulate oncogenic properties of cells in vitro (27,28). HCMV illness can arise by reactivation of an existing strain or reinfection with a new strain (29). The newly available data show that CMV is found in a variety of breast cancer patient samples (30).

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According to (31), HCMV infection may enhance some of the characteristics that are the hallmark of cancer. These characteristics include dysregulation of the cell cycle, suppression of apoptosis, and increased migration and invasion (28,32).

The association between EBV or HCMV and cancer such as breast and prostate cancer risk shows contradictory results and it is still difficult to determine whether both or none of the viruses are causally associated with cancer (33–35), contradictions are believed to arise from the various methodologies employed (36). In addition, EBV types occur worldwide, but in their geographic distribution, they differ. Positive correlations between these viruses and breast cancers have been shown using different techniques, especially polymerase chain reaction (PCR) (33).

2. Methods

Group study

A sampling of formalin-fixed and paraffin-embedded specimens

This study included a total of (45) breast tissue samples that were formalin-fixed paraffin-embedded (FFPE). Of theste, (33) FFPEs had breast cancer tumors, and (12) FFPEs with benign breast tumors were regarded as the control group in this study. These patients are divided into 2 age groups 19-40 and 41-65 years old. Tumor type was Invasive ductal Carcinoma (IDC) in (81.8%) of the cases, Invasive lobular Carcinoma (ILC) in (9.1%) of the cases, and other types in (9.1%) of the cases. In addition, the malignant tumors were identified in 7/33 (21.2%), 5/33 (15.2%), and 21/33 (63.6%) individuals, respectively, at grade I, grade II, and grade III. All of the samples were collected between November 2020 and May 2021 at Al - Fayhaa Teaching Hospital and Al-Sadder Teaching Hospital in the ALBasrah region of Iraq to identify EBV and CMV using conventional PCR.

The Process of Extracting Viral DNA

Using the ReliaPrepTM FFPE gDNA Miniprep System DNA Extraction Kit (Promega / USA), total DNA was extracted from formalin-fixed paraffin-embedded tissue. The DNA was then prepared by the manufacturer's guidelines. Before extracting DNA, the embedding material must be completely removed from the 5 µm- thick tissue samples made from paraffin-embedded blocks. The tissues were first deparaffinized and rehydrated with mineral oil and alcohol, then lysed with proteinase K and tissue lysis buffer. Following the manufacturer's instructions, DNA was extracted from lysed tissue, The Nanophotometer was used to measure the eluted DNA samples and the nucleic acids were then frozen at -20°C.

Polymerase Chain Reaction (PCR)

Two viruses, and GAPDH enzyme DNA, were successfully extracted from paraffin-embedded tissues from both breast cancer and benign breast tumors. All samples were subjected to conventional PCR employing primers for detection Table (3-4), (3-5), and (3-6). According to the PCR conditions (Table3-8), About 5 µl of crude DNA extract was added to a reaction mixture (Promega, USA) which is 25 µl in volume, the products were amplified.

Table (3-4): Conventional PCR primer sequences for EBV.

Primers		Sequence	Product size	Reference
EBNA 1	Forward	GTCATCATCATCCGGGTCTC	269 pb	(37)
	Reverse	TTCGGGTTGGAACCTCCTTG		
LMP-2	Forward	CTAGCGACTCTGCTGGAA AT	307 or 337 bp	(38)
	Reverse	GAG TGT GTG CCAGTTAAGGT		
EBNA-4	Forward	CCGAAGAGGTTGAAAACAAA	230pb	(39)
	Reverse	GTGGGGGTCGTCATCATCTC		
GAPDH	Forward	GGC CTC CAA GGA GTA AGA CC	157 pb	(38)
	Reverse	CCC CTC TTC AAG GGG TCT AC		

Table (3-5): EBV genotyping primers sequence used in Nested PCR.

Primers		Sequence	Product size	Reference
EBNA3C	Forward	AGAAGGGGAGCGTGTGTTGT	Type 1:153 Type 2:246	(40)
	Reverse	GGCTCGTTTTTGACGTCGGC		

Table (3-6): Conventional PCR primer sequences for human cytomegalovirus (HCMV).

Primers		Sequence	Product size	Reference
UL55	Forward	GGTCTTCAAGGAACT CAGCAAGA	72 pb	(41)
	Reverse	CGGCAATCGGTTTGTTGT AAA		
IE2	Forward	TCCTCCTGCAGTTCG GCT TC	240 bp	(42)
	Reverse	TTTCATGATATTGCGCAC CT		
HindIII-X	Forward	GGATCC GCATGGCATTCACGTATG T	406 pb	(43)
	Reverse	GAATTCAGTGGATAACCTGCGGCG A		

Table (3-8) :PCR conditions for genes amplification.						
Genes	Initial Denaturation °C/ min.	Denaturation °C/ sec	Annealing °C/ sec	Extension °C/ sec	Final extension °C/ min	cycles
UL55	95 /7	94/30	63 / 30	72 / 30	72 /7	35
HindIII-X	95/10	95 /30	55/60	72/60	72 / 10	30
IE2	95 /10	95 /60	50 /60	72 / 60	72 /10	40
GAPDH	96 /2	96/30	55 /60	72 / 120	72/ 10	39
EBNA 1	94 /10	94 /90	60 / 60	70 / 120	72°/ 10	30
EBNA-4	94 /5	94/30	55 / 60	72 / 90	72/5	30
LMP2	94 /5	94 /30	55 / 60	72 / 90	72/ 5	30
EBNA3C	94 / 1	94 /30	51 /60	72 /30	72 /5	20

3. Results

The age group of 41-65 years old showed the highest incidence of breast cancer in this study. This study shows that the highest frequency of breast cancer (64.4%) was in women with age 41-65 years. Gathered, comprising: (33) malignant tumors with a mean of (48.2) and (12) benign tumors with a mean of (31.7), the mean age of patients with malignant tumors was higher than the mean age of the benign tumor patients. A significant difference ($p< 0.05$) was found between them according to age. Invasive Ductal Carcinoma (IDC) was the most prevalent histopathological type, while high-grade tumors were the most prevalent grade table (4-1).

Table (4-1): The distribution of the study population by age, histological type, and grade.	
Characteristic	Percentages
Age group	
19-40 y	8 (24.2 %)
41-65 y	25 (75.8%)
Histological types of breast cancer	
Invasive ductal carcinoma	27 (81.8%)
Invasive lobular carcinoma	3 (9.1%)
Others (invasive intraductal carcinoma, infiltrating ductal carcinoma, and intraductal papilla adenocarcinoma)	3 (9.1%)
Grade	
GI	7 (21.2%)
G II	5 (15.2%)
G III	21(63.6%)
Histological types of benign tumors	
Fibrocystic	5(41.7%)
Fibroadenoma	4(33.3%)
Lipoma	3(25%)

Molecular detection

For GAPDH, EBV, and HCMV, as well as genotyping for EBV, Molecular detection has been carried out. Total DNA was first extracted, followed by PCR-amplification of the extracted DNA and the PCR products were then run via a gel electrophoresis procedure.

An agarose gel revealed PCR results as a distinct band. Before finding the virus' DNA, GAPDH primers were used to find human DNA in the cell lysate from both malignant and benign breast tumors. All benign and malignant breast tumors had human GAPDH DNA that could be successfully detected and amplified, and the product size was 157 bp (Figure 4 - 2).

Detection of EBV DNA

A total of 45 tissue samples were diagnosed with EBV DNA (EBNA1). Among the 33 breast carcinoma,19/33 (57.6%) cases showed positive results while among 12 benign breast tumors control group was EBV DNA positive 6 (50%). There was a significant difference between breast cancer patients and benign tumors (pv0.009). The result of the PCR assay showed an increasing percentage of EBV DNA (EBNA1) in breast carcinoma patients age group 41-65 years 15 (45.5%) compared with the benign tumor age group the highest percentage in the age group is 19-40 years 4(33.3%). According to the histopathological types and grades, EBV is positive with a high prevalence in IDC and with G III as shown in tables (4-2) and (4-3). While there was no positive LMP2 in benign breast tumors, the positive sample for LMP2 was 2 of 33 (6.1%) in breast cancer samples. None of the 45 samples tested positive for the EBV (EBNA4) gene.

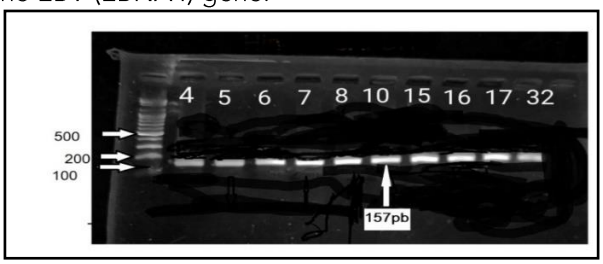

Figure (4- 2): Agarose gel electrophoresis at (70 V) for (1 hour) of PCR amplified products of GAPDH. First left lane = DNA ladder (100-1500 bp), Lane 1-10 = positive sample.

Table (4-2): Frequency of EBV infection among histopathological types.			
Histological types	Total	EBV positive	Negative
Invasive Ductal Carcinoma	27	16	11
Invasive Lobular Carcinoma	3	2	1
Others types	3	1	2

Table (4-3): The detection of (EBV) infection according to grade.			
Grade	Total	EBV positive	Negative
I	7	5	2
II	5	3	2
III	21	11	10

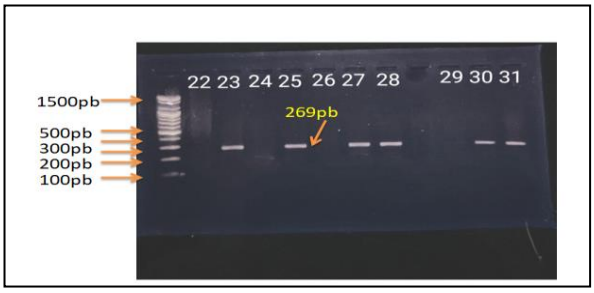

Figure (4-3): Conventional PCR detection of the EBV EBNA-1 gene. The figure explains the presence of the EBV EBNA-1 gene at molecular position 269bp, the positive result in the lines (2,4,6,7,10 and 11) and the identification of viral DNA by PCR in ethidium bromide-stained (2 % agarose gel, 60 minutes at 70 V).

EBV Genotypes among breast tumor patients.

The second PCR was performed using the amplified product of the first round PCR, by EBNA-3C primer was used to distinguish between type 1(153pb), and type 2(246pb). 25 positive EBNA1 samples19 in breast cancer and 6 in benign tumors in the first round and genotyped in the second round by (Nested PCR) using the EBNA3C gene.

The present results showed that EBV type 1 was detected in all 25 samples that were proved positive for EBNA1 by nPCR.EBV type 2 was not found in any of the samples processed. The application of genotyping PCR to subtype EBV revealed the circulation of only one type (type 1) in the breast sample.

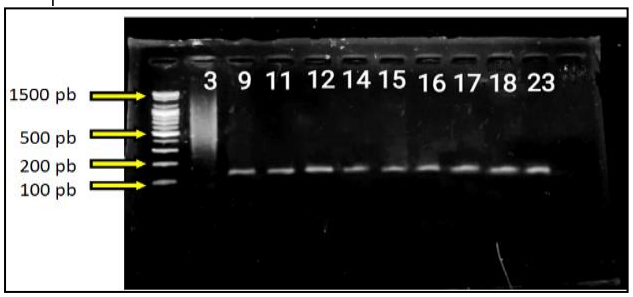

Figure (4-5): EBNA3C-specific primer was used in PCR amplification for the samples' positive EBNA1 gene results. A 153 bp fragment was amplified by samples of genotype 1. (2% agarose gel, 70 V, for 1 hour) was used. nPCR products stained with ethidium bromide and seen under ultraviolet light. M: 100 bp DNA marker; EBNA3C is present in lanes (2, 3, 4, 6, 7, 8, 9, and 10).

Detection of HCMV

The findings of the PCR technique found HCMV DNA in breast cancer, 31/33 (93.9 %), and 11/12 (91.7 %) of benign breast tumors using the UL55 gene Figure (4-6). The prevalence of UL55 between breast cancer and benign tumors showed a significant difference (pv0.002) in this investigation. In this recent study, the highest prevalence of HCMV infection in breast cancer with (IDC) was 26 out of 27and G III 20 out of 21, while the high frequency of HCMV infection in benign tumors was a fibrocystic type 5 out of 5, as shown in table (4 - 4) (4- 5) and (4- 6):

Table (4- 4): Frequency distribution of histopathological typing of breast carcinoma and Human cytomegalovirus (HCMV) infection.			
Histological subtypes	Total	HCMV positive	Negative
Invasive Ductal Carcinoma	27	26	1
Invasive Lobular Carcinoma	3	3	0
Other types	3	2	1

Table (4-5): The distribution of the study population by Grade and HCMV infection.			
Grade	Total	HCM positive	Negative
I	7	7	0
II	5	4	1
III	21	20	1

Table (4- 6): Frequency distribution of histopathological typing of benign breast tumor and HCMV infection.			
Histological types	Total	HCMV positive	Negative
Fibrocystic	5	5	0
Fibroadenoma	4	4	0
Lipoma	3	2	1

The results were considered positive if a molecular band after gel electrophoresis was present in the specific position, for HCMV, a specific primer was used to detect the UL55 gene with a predicted PCR product of about 72 bp figure (4-6).

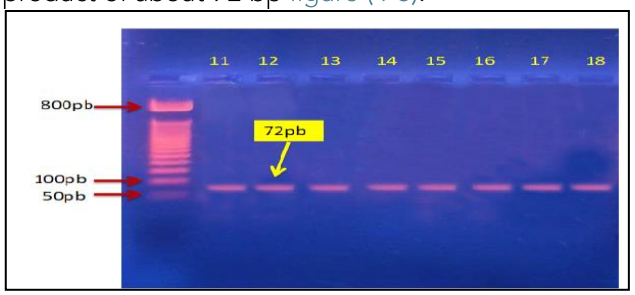

Figure (4-6): Detection of HCMV UL55 Gene by Conventional PCR. Gel electrophoresis for amplified UL55gene, PCR detection of virus DNA in ethidium bromide-stained (2% agarose gel, 50 minutes at 70 V). The image explains the presence of HCMV UL55gene at molecular position 72bp, the positive results are present in lines (1, 2, 3, 4,5,6,7 and 8).

Using conventional and gradient PCR, none of the 45 samples tested positive for the HCMV IE2 gene and HindIII. The expected size of the

Immediate Early (IE) gene amplicon was 240bp while the HindIII-X fragment gene was 406bp in size.

Detection of both viruses in the same sample

The study revealed the presence of both viruses in the same sample with a percentage of (51.1%) in 23 cases out of 45.

4. Discussion

In the current study, the genomes of the HCMV and the EBV were examined in 45 samples of FFPE breast cancer and benign tissue (33 breast cancer and 12 benign tumors) with ages ranging from 19 to 65 years and a mean of (43.8%). The majority of them were in the age group 41-65 for breast cancer and 19-40 for benign. The current findings are consistent with those reported globally, where these breast malignant tumors typically affect women over the age of forty (44,45).

Current results are in agreement with those that have been published globally, where these breast malignant tumors often affect women over the age of forty (46). These findings would suggest that aging is a significant risk factor for tumor alterations that arise in lesions of the breast epithelial tissues. Malignant alterations in breast epithelial tissues tend to occur more frequently as people age, and as a result, their frequency has been reported to rise with age (46,47).

In the present study, the breast cancer tissue included 27 cases (81.8%) of IDC, 3 cases (9.1%) of ILC, and three (9.1%) samples of invasive intraductal carcinoma, Infiltrating ductal carcinoma, and intraductal papil adenocarcinoma. The most common form of breast cancer in the current study was IDC. This current result is consistent with another local study in Basrah Governorate (48). El Shazly et al (2018), likewise found that Invasive ductal breast carcinoma was the most common histological type in their investigation (49,50), which is consistent with the findings of the current study. Bharat et al (2009) also reported the same findings, which corroborates these findings (51). In 2021, there are 281,550 new instances of invasive breast cancer identified in women in the USA, with IDC accounting for the majority of these cases (80%) (52).

Screening for EBV genome in breast cancer tissue

Several observations offer intermittent proof of an association between EBV and breast cancer including; (a) EBV is present in breast tissue, where it has been reported in some women's breast milk (53); (b) human breast milk cell development is stimulated by EBV DNA transfection (54);(c) Breast cancer is a location of some EBV-associated lymphomas (55,56);(d) However, there is data that implies that primary EBV infection rather than viral oncogenesis is the cause of breast cancer (57), it shares epidemiological features with young-adult Hodgkin's lymphoma). It has been shown that EBV is present in the benign breast cancers of immunocompromised women (58);(f) Breast epithelial cells can acquire EBV by direct interaction with EBV-bearing lymphoblastoid cell lines in-vitro (59).

About half of the breast tumors (19/33, or 57.6%) we investigated had the EBV genome, and of the 12 benign breast tumor control groups, only six of them had positive results (50 %). and, in particular, in those of the most common form, invasive ductal carcinoma (IDC).

The current study is consistent with a previous study from Al-khalidy and Mukhlis, (2010) showed that the amplified EBNA-1 bands of EBV were detected in 26/50 (52 %) of the malignant group, but the amplified LMP-1 bands were detected in only 10/50 (20 %) of the malignant group (60). This outcome was also in line with a study conducted in Jordan in 2013 by Khabaz, 2013. The findings revealed that 24 out of 92 (26%) breast cancer specimens tested positive for EBV, compared to 3 out of 49 (6%) samples from the control group. This difference was statistically significant (38).

According to the present results of the PCR assay, patients with breast cancer aged 41 to 65 years revealed an increasing percentage (45.4%) of EBV DNA (EBNA1), while the highest percentage of benign found in the age group of 19 to 40 years (33.3%). The increased incidence of breast cancer with age supports the theory that breast cancer is caused by a virus. If late breast cancer virus exposure represents one 'hit,' the longer a woman lives, the greater her chances of receiving a second 'hit' and getting breast cancer (61).

According to the current study, high-grade tumors were present in the majority of EBV-positive tissues 11 /21. We can thus assume based on our findings and those of other studies that there is a connection between the presence of EBV and the grade of malignancies (18,62,63).

Several studies have demonstrated a positive association between EBV and breast cancer using PCR-based methods (63,64). Different parts of the world have varying rates of EBV detection in breast cancer. In one of these studies, 4/35 (11.42 %) of the control group's fibro adenoma samples and about 10/37 (27.02 %) of the samples of ductal breast cancer tested positive for EBV DNA (p=0.095).

Despite numerous studies that demonstrated the presence of EBV genetic material in up to 51% of breast cancers, the identification of the EBV genome in breast carcinoma and its role as a carcinogen has been bitterly debated over the past decade (21,65,66). This discrepancy can be attributed to certain researchers' failing to detect EBV in breast cancer (66).

Although there have also been reports of negative results, the PCR investigation by Gaffey et al. (1993) failed to detect EBV in any of the 18 infiltrating breast carcinomas or the 16 medullary carcinomas (67).In Iran, 300 breast biopsy specimens were analyzed, and PCR assays were performed but did not report

any genomic DNA fragment of EBV (68).
In the current study, only 2 out of 33 (6.1%) breast carcinomas tested positive for LMP2, while no benign breast tumors tested positive. The EBV(EBNA4) gene was not found in any of the 45 samples. These findings are indeed by Speck et al. (2003) who used PCR with primers specific for the LMP2 and BHRF1 viral genes to check 22 breast cancer-derived cell lines for the presence of EBV DNA. In these cell lines, no EBV DNA was amplified (69).
However, the current findings contradict Bonnet and colleagues, who found EBER2 and LMP2 DNA by polymerase chain reaction (PCR) in 51 of 100 breast tumors. Only 10% of these specimens had EBV DNA that could be detected when normal breast tissue from the same individuals was tested (62).
The present findings also conflict with those of a Sudanese investigation that detected the EBV genome in 49 (53.3%) and 10 (11%) individuals, respectively, using LMP-1 and EBNA-4 PCR. EBV was detected in 12 (24%) of the patient's control tissues when LMP-1 primers were used, but none of the control samples tested positive when EBNA-4 primers were used (70).

EBV Genotyping by (EBNA-3C) using Nested PCR Amplification

The current finding revealed the circulation of only one subtype (type 1) in the breast sample. The genotype is more detected with IDC type (48.5%) and in stage 3 (33.3%). The current findings are consistent with a study published in Ahvaz, Iran in 2019 by Sharifpour et al.,(2019) which revealed that seven positive samples belonged to EBV type 1(71). This finding is also consistent with the findings of studies conducted in Pakistan, which found that 66.66 % of patients had EBV-1 whereas just 1.66 % of patients tested positive for EBV-2 infection (72). Salahuddin et al., (2018) found that all types of lymphoma populations from various hospitals in Islamabad, Pakistan, had the EBV-1 genotype as the predominate genotype (73). According to Tabibzadeh et al., (2020) in the Iranian population (91.2%) and Janani et al. 2015 among Indians, the most common genotype was EBV-1 100 % (74,75).

Screening for HCMV genome in breast cancer tissue

The virus enters the host cell through membrane fusion, facilitated by HCMV envelope glycoproteins attaching to host cell receptors (s). Multiple membrane glycoproteins are necessary for HCMV entrance (76). Glycoprotein B (gB) facilitates HCMV entrance by serving as a fusion protein, essential for viral entry and cell-to-cell transmission but not attachment (77). The current research used FFPE to amplify the HCMV gB (UL55), HindIII-X fragment genes, and IE2 genes. In the current investigation, the Human Cytomegalovirus UL55 gene was found in 31 of 33 cases (93.9%) of breast cancer and 11 of 12 benign tumor controls (91.7%).
The difference in CMV DNA detection between malignant (case) and benign (control) tumors was significant (pv= 0.002). The virus was found in 7%, 4%, and 20% of grades I, II, and III, respectively. The majority of HCMV-positive cases (n=27) were found in invasive Ductal carcinoma, The same results were also reported by Tsai et al., (2005), who found that 47 out of 62 cases (76%) of Cytomegalovirus associated breast cancer were invasive ductal breast carcinoma (42).
According to current findings, none of the 45 samples tested positive for the HCMV IE2, HindIII-X fragment gene, which is consistent with another investigation in 2019 in Sudan (78). However, the current findings differ from those of Tasi et al., (2005), who found HCMV DNA in 47/62 (75.8%) of the samples using the IE2 gene (42).
This high prevalence of HCMV DNA could be attributed to the detection of latently infected cells or extracellular DNA fragments in patients who were not infected at the time of testing. This is in line with earlier studies that showed similar quantitative outcomes (79–81).In contrast to the detection of DNA for immediate early genes (IE), a study by Yang et al., (2022) that employed nested PCR to detect HCMV glycoprotein B (gB) DNA in breast cancers was found to be sensitive and specific. In this study, HCMVgB DNA was found in 18.4% of 136 breast tumors, and 62.8 % of 94 patients with breast cancer were HCMV seropositive (82).
The HCMV immediate early gene mRNA (IE2) was not found in any of the samples, indicating viral latency in breast cancers. Moreover, Mengelle et al., (2003) found nearly identical results when using whole blood, which supports the current findings (83).
Al-Baiati et al., (2014) published another study in which the Polymerase chain reaction (PCR) technology was employed to amplify two viral genes (UL55 and UL97) within host genes. According to the results of polymerase chain reaction, 89% and 77% of infertile women and 87% and 67% of breast cancer, respectively, were positive for these genes(41). Current results were also supported by Taher et al., (2013) who found that HCMV DNA was abundantly detected in 100% of breast cancer specimens (15).
In addition, in Iran, Karimi et al., (2016) detected HCMV DNA genomic fragments in 26 of 50 (24.75 %) invasive breast ductal carcinoma (84). Investigations have revealed that cancer does not require the presence of all viral antigens. The HCMV immediately early antigen (IE Ag) has often been considered a target antigen by IHC since it is known to be essential for controlling the expression of other viral genes, replication, and cell transformation (85,86).
Results from various methodologies generally show that HCMV is highly prevalent in Asian and African countries while being relatively less prevalent in European and South American countries. Numerous studies have shown that low income and social status

in third-world nations are connected to the increased prevalence of HCMV (87,88).

The issues with working with FFPE samples also include the small amount of extracted genomic DNA and DNA fragmentation (89). It may be inferred that many factors, including geographic location, EBV infection detection techniques, sample type, detection regions or genes in the HCMV and EBV genomes, and the histological types of breast cancer, may contribute to the heterogeneity in results between researchers.

Detection of both viruses in the same sample

The study revealed the presence of both viruses in the same sample with a percentage of (51.1%) in 23 cases. Infection by two or more viruses may also be required for the development of breast cancer (90,91).

This finding raises the possibility that multiple viral infections may be linked to both benign and malignant breast cancer. In conclusion, HCMV and EBV may contribute to the development of breast cancer. Viral co-infection may be a significant mechanism contributing to the induction of breast cancer as evidenced by the high incidence of viral co-infection in breast cancer tissue. Multiple viral infections may raise the chance of developing breast cancer in women.

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