ORIGINAL ARTICLE

Human Cytomegalovirus and COX$_2$ Expression among Women with Breast Tumors

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ABSTRACT

Background: *The human cytomegalovirus (HCMV) has been linked to all cancer characteristics, leading to increasing speculation that it plays a role in oncomodulation and human carcinogenesis. An increasing body of research demonstrates a connection between HCMV infection and a number of cancers, including breast cancer. Inflammatory cells produce chemokines, cytokines, and growth factors into the microenvironment of the tumor that encourage tumor growth and malignancy. Biopsies of breast cancer often showed COX-2 expression, which HCMV might be the cause of.* ***Objectives:*** *Our study aimed to through a beam of light on HCMV as a viral etiology of breast tumors and investigate the association between HCMV infection and simultaneous expression of COX2 in women with breast tumors.* ***Methodology:*** *We assessed HCMV DNA presence by nested type PCR and COX-2 by ELISA in breast tissues obtained from 33 breast cancer (BC) patients, 24 benign breast lesions, and 33 normal breast tissues.* ***Results:*** *We found that there was a statistically significant weak association between HCMV DNA in malignant, benign breast lesions and normal breast tissue groups (p=0.036) and (V =0.283). Also, there was a statistically significant difference between the studied malignant, benign breast lesions and normal breast tissue groups as regard median COX-2 (p =0.0088). In addition, presence of HCMV proteins (E/IE) was identified in 4 breast cancer tissue samples (25%), there was a weak positive correlation between HCMV viral proteins (E/IE) and COX-2 in group IA with statistically insignificant difference r= 0.26 and (p= 0.15).* ***Conclusion:*** *We concluded that the existence of CMV DNA and proteins in breast cancer (BC) tissues points to the virus's involvement. In addition, COX-2 may contribute to the development of BC.*

INTRODUCTION

Globally, breast cancer (BC) stays the primary reason of mortality for women. There are multiple known breast cancer development risk factors, including early menarche, late menopause, age, sex, nulliparity, hormone replacement treatment, and mutations in the *BRCA1* and *BRCA2* genes. Furthermore, infectious pathogens are now recognized as a major element in cancer development [1]. More than 22,000 new cases are diagnosed each year, accounting for 33% of all female cancer cases in Egypt [2].

Investigations have linked a variety of viral infections related to breast cancer, including human papillomavirus, human mammary tumor virus, Epstein–Barr virus (EBV), polyomavirus, bovine leukemia virus, and human cytomegalovirus (HCMV) [3]. Human cytomegalovirus, called human herpesvirus 5 as well, is a virus that causes acute or chronic latency and persistence and affects 70–90% of people worldwide. Although HCMV infections are typically asymptomatic, serious illnesses are more likely to arise from them in immune-compromised people [4].

Despite the discovery of HCMV Proteins and DNA in tissues of breast cancer, usually, the virus is not regarded as an oncogene [5]. In cancer patients, CMV reactivation is considered another interesting aspect in the virus's carcinogenic capacity [6]. Proteins that HCMV encode control responses of adaptive immune system to escape immune recognition and avoid elimination in its host. In addition, it can promote all the steps of hallmarks of cancer, for instance inhibition of apoptosis, cell cycle dysregulation, increased migration, invasion, plus immune evasion [5].

Documentation suggests that during the latent infection phase, the proteins pp65, IE1, and US28 expressed by HCMV genes may accelerate some

cancers growth. HCMV-encoded proteins regulate adaptive immune responses, allowing the virus to evade immune detection and host eradication [7]. When an infection is active, the most prevalent protein found in virion protein and non-infectious viral particles is called PP65. Certain gene products of HCMV, such as immediate early proteins (IE1) and IE2, which are known to stimulate S phase entry, can have profound effects on cell development [8]. Evidence has indicated that US28, another HCMV gene with constitutive signaling activity, activates the genes for cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), and Signal Transducer and Activator of Transcription 3 (STAT3) [9-10].

Within the tumor microenvironment, inflammatory cells release growth factors, chemokines, and cytokines that encourage tumor growth and malignancy [11]. Breast cancer biopsies often showed COX-2 and 5-LO (5-lipoxygenase) expression, which could be provoked by HCMV [12].

Forty percentage of patients with invasive breast cancer had COX-2 overexpression, and this was connected with a poor prognosis [13]. Thus, COX-2 inhibitors are being researched as possible novel cancer treatments [14].

METHODOLOGY

The study's protocol was approved by Institutional Review Board (IRB), Faculty of Medicine, Mansoura University; code number: MD. 21.08.506.

This case-control study was carried out over a period of 2 years, from September 2021 to August 2023. Ninety female patients were selected from the Surgical Units at Oncology Center. Ninety breast tissue samples were obtained, transported, and processed in the virology lab at Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University.

The cases were laboratory classified into: (**Group I**); we assessed 33 breast cancer patients for the existence of HCMV in fresh cancerous tissues (**Group IA**), Additionally, 24 benign breast tumors' fresh tissues had their HCMV levels evaluated (**Group IB**). A control group of thirty-three breast tissue samples taken from women in good health (**Group II**). Extraction of DNA was done using QIAamp® DNA Mini kits (**Qiagen, Germany**). All extraction procedures were done according to manufacturer's protocols. The concentrations and purity of all extracted DNA were measured using the NanoDrop™ 2000/2000c spectrophotometer (**Thermo Scientific, USA**).

Initially, each DNA sample was subjected to PCR with consensus primers PC04/ GH20 (β-globin) (**Table 1**) to confirm the quality of the DNA extracted (used as an internal control), by the use of DNA thermal cycler (**PTC- 100**), after an initial denaturation step at 95°C for 3 min, 45 cycles were programmed as follows: The steps are as follows: 30 seconds of denaturation at 95°C, 40 seconds of annealing at 53°C, 40 seconds of primer extension at 72°C, and 5 minutes of final extension at 72°C [15].

Every sample of breast tissue was examined to check for the existence of immediate early (*IE*) gene by nested type PCR using primers illustrated in **table 1**. A 15-minute initial denaturation stage at 95°C was followed by 40 cycles of 30 seconds at 95°C, 3 minutes at 60°C, and 1 minute and 30 seconds at 72°C in order to complete the amplifications. The second PCR DNA template was five μl of the first PCR result. The number of cycles was reduced to 30, but the methodology remained the same as in the previous round. Following amplification, aliquots were examined by means of gel electrophoresis composed of 1.5 % agarose [16].

Table 1: Primers that were used for detection of *β-globin* and IE genes by PCR [15-16]

Target	PCR round/ Gene	Primer sequence (5′ -3′)	Amplicon size (bp)
β-globin Used as an Internal Control for DNA Extraction.	PC04	F- CAA CTT CAT CCA CGT TCA CC	268
	GH20	R- GAA GAG CCA AGG ACA GGT AC	
CMV/IE	First	F- CCAAGCGGCCTCTGATAACCAAGCAGCC	435
		R- CAGCACCATCCTCCTCTTCCTCTGG	
	Second	F- AGTGTGGATGACCTCGGGCCATCG	110
		R- GGTGACACCAGAGAATCAGAGGAGC	

The enzyme linked immunosorbent assay (ELISA) kit supplied by (**Shanghai Korain Biotech Company, China**) was employed to measure the COX-2 level in breast tissue homogenate. Paraffin cancerous breast tissues (**Group IA**) were used for immunohistochemistry (IHC) in order to detect the presence of HCMV protein, wherein the primary antibody (CCH2 + DDG9) (**Dako, Denmark**) was used.

Statistical analysis
Software used: R 4.2.3 was utilized for data analysis.
- Numerical variables: the median and interquartile range were explained. When analyzing categorical variables, frequency and percentage were utilized. The Kruskal-Wallis rank-sum test and the Wilcoxon rank-sum test, followed by Dunn's post hoc test, were used for comparison of non-parametric numerical variables. Fisher's exact test and Pearson's Chi-squared test were used for comparison of the changes in the categorical variables. Cramer's V was used to quantify the degree of correlation between two nominal variables.
- The Spearman's rank correlation coefficient (rho) was used to calculate the degree of correlation between two numerical variables. Every test had two tails. A

95% confidence interval (CI) accustomed to determine statistical significance for p-values less than 0.05.

RESULTS

The studied patients in group IA aged from 24 to 73 years with mean ± SD 52.52±11.93. In group IB, age ranged from 35 to 53 years with mean ± SD 44.88±5.29. Group II patients' ages ranged from 32 to 51 years, with mean ± SD 40.24±5.72. There was a highly statistically significant difference between the studied patient's groups regarding age (p value < 0.001), as shown in **table 2**.

Table 2: Age distribution among the studied groups:

Group	Sub-group	Description	N (%)	Age (years) Mean ± SD	P value
I (Cases)	**IA**	Breast cancer patients	33 (36.7%)	52.52±11.93	
	IB	Patients with benign breast lesions	24 (26.6%)	44.88±5.29	**<0.001**[*]
II (control)		Patients who underwent reduction mamoplasty breast surgery	33 (36.7%)	40.24±5.72	

N= number, SD= standard deviation, significant p <0.05.

Invasive lobular carcinoma (ILC) was present in three patients (9%), one case (3%) had high grade ductal carcinoma, and 27 cases (82%) of the 33 breast cancer samples evaluated in group IA had invasive duct carcinoma (IDC) as their diagnosis. One case (3%) had undifferentiated carcinoma and one case (3%) had mixed ductal mucinous carcinoma in situ. Grade II accounts for 78.8% of breast cancer cases, with grade III accounting for 18.2% and grade I account for 3%. The TNM AJCC 8th edition was used to stage carcinomas. In the T2 and T3 categories, there were 90.9% and 9.1% of cases, respectively. 48.5% of cases had positive lymph nodes, while 42.4%, 6.1%, and 0% of cases had N1, N2, or N3 stages visible. Human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR), besides estrogen receptor (ER) positive were detected in 100%, 84.8%, and 12.1% of the cases, respectively. There were 39.4%, 21.2%, and 39.4% of cases with Ki-67 reactivity ranging from 0–16%, 16–30%, and > 30%, respectively.

In group IB, 1 phyllodes tumor accounted for 4.2% of all benign breast lesions, whereas 21 cases (87.5%) had fibroadenoma diagnosis, 2 cases (8.3%) had lipoma diagnosis. Normal breast tissues extracted from female breast tissues following conservative breast surgery were incorporated in Group II.

As seen in **Figure 1**, the beta-globin (β-globin) gene was found at 268 bp fragment in all samples of breast tissue, including benign, malignant, and normal tissues. Nested type PCR for immediate early (IE) gene was done. In group IA, four samples (12%) were positive for this gene after the 2nd round of PCR at 110 bp, as shown in **Figure 2**. This gene wasn't detected in groups II and IB. Only 4 out of 33 female breast cancer patients (12%) in group IA had the IE gene identified. However, groups II (normal breast tissues) and IB (benign breast tumors) did not have this gene. There was a statistically significant weak association between HCMV *IE* gene and the three different groups (Group IA, IB, and II), (p= 0.036) and (V =0.283) as presented in **table 3.**
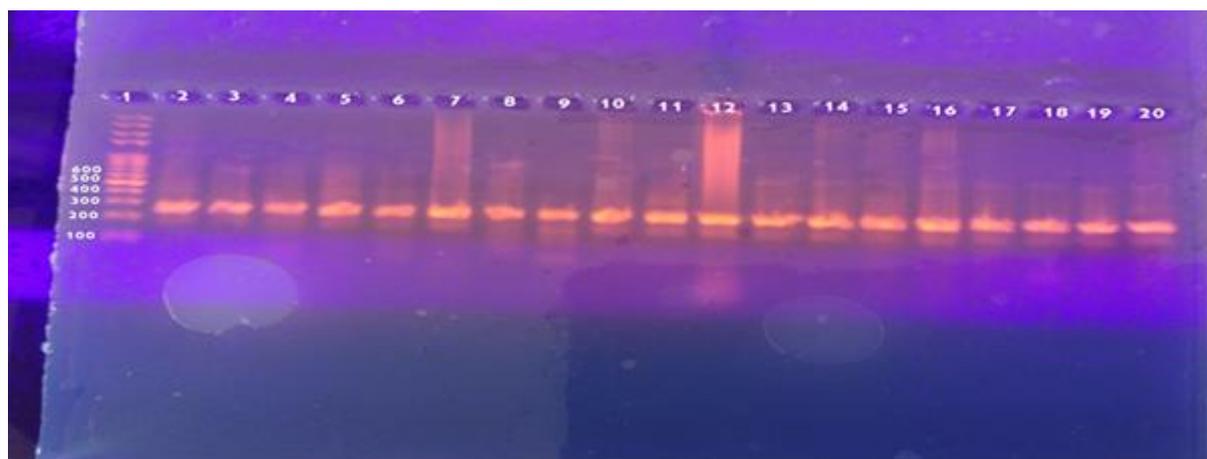
Fig. 1: Agarose gel electrophoresis for *β-globin* amplicons in breast tissue samples.
Lane 1 represents 100-bp DNA molecular size marker, **lane 2-20** represents PCR products for β-globin gene (268 bp).
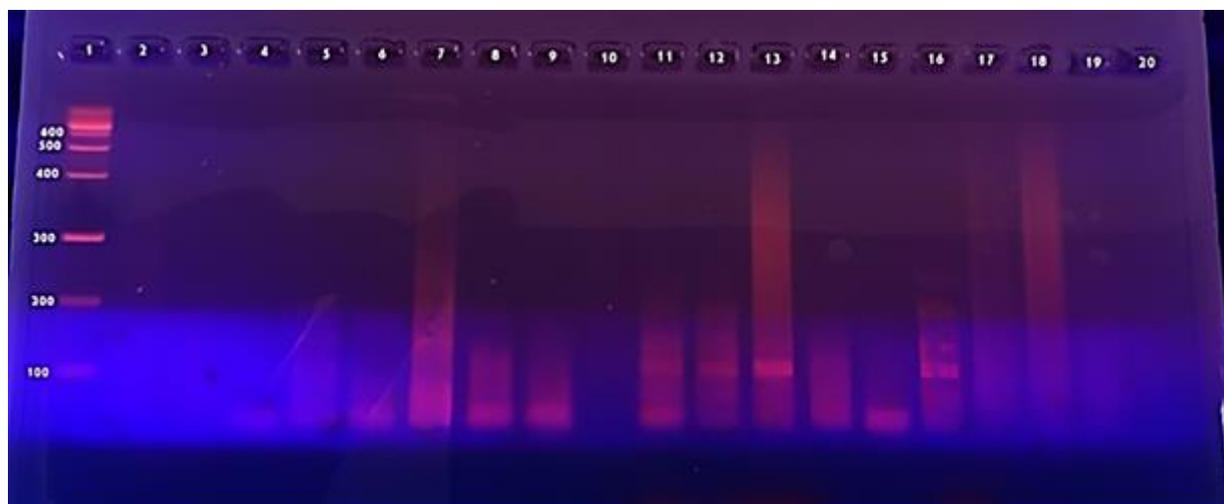
Fig. 2: Agarose gel electrophoresis for CMV/IE amplicons in group IA.
Lane 1 represents 100-bp DNA molecular size marker, **lanes 11, 12, 13 and 16** are positive samples for *IE* gene (110 bp).

Table 3: Association between cytomegalovirus immediate early and glycoprotein genotypes between the studied groups:

Group	Description	Samples without detected HCMV genes, N = 86	Samples with detected HCMV genes , N = 4	p-value	Cramer's V
IA	Breast cancer	29 (34%)	4 (100%)	**0.036[*]**	**0.283**
IB	Benign breast lesions	24 (28%)	0 (0%)		
II	Control	33 (38%)	0 (0%)		

N: Number, HCMV= human cytomegalovirus, significant p $<$ 0.05.

As shown in **table 4**, there was a statistically significant difference in the median COX-2 value between the groups under study, with group IA having a higher median value than groups IB and II (p = 0.0088)**.**

According to the results of the Dunn's post hoc test, COX-2 expression in breast tissues varied statistically between groups IA and II (p-value = 0.024) and IB (p-value = 0.027).

Table 4: Comparison of COX-2 between the studied groups:

Group Characteristic	Group IA Breast cancer, N = 33	Group IB Benign breast lesions, N = 24	Group II Control, N = 33	p-value
COX-2 ng/L Median (IQR)	200 (90-600)	90 (79.5-120)	90 (89-110)	0.0088*

N: number, IQR= interquartile range, significant p $<$ 0.05.

In group IA, four breast cancer cases tested positive for the HCMV E/IE protein. Out of four samples, one (25%) exhibited cytoplasmic and perinuclear immunostaining score II, and three (75%) had cytoplasmic immunostaining score III, as illustrated in **Figures 3 & 4**.
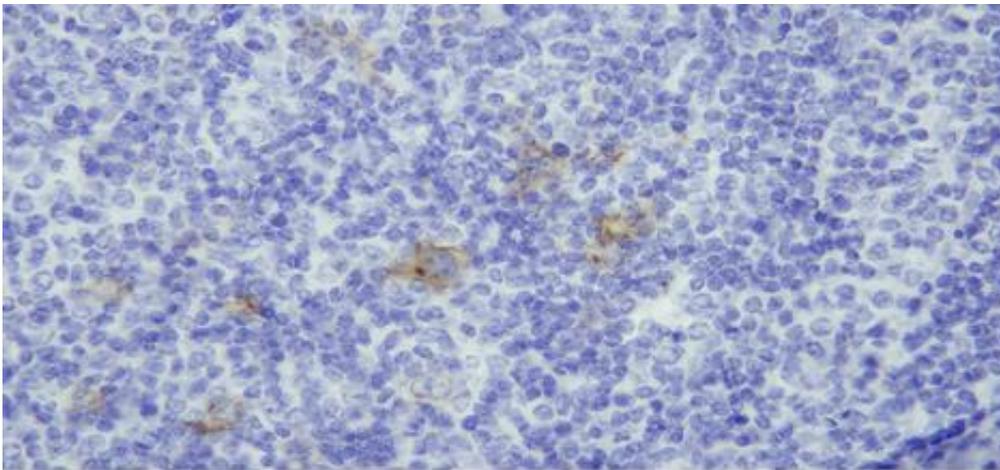
Fig. 3. A case of invasive ductal carcinoma GII with positive perinuclear immunostaining (Score II)
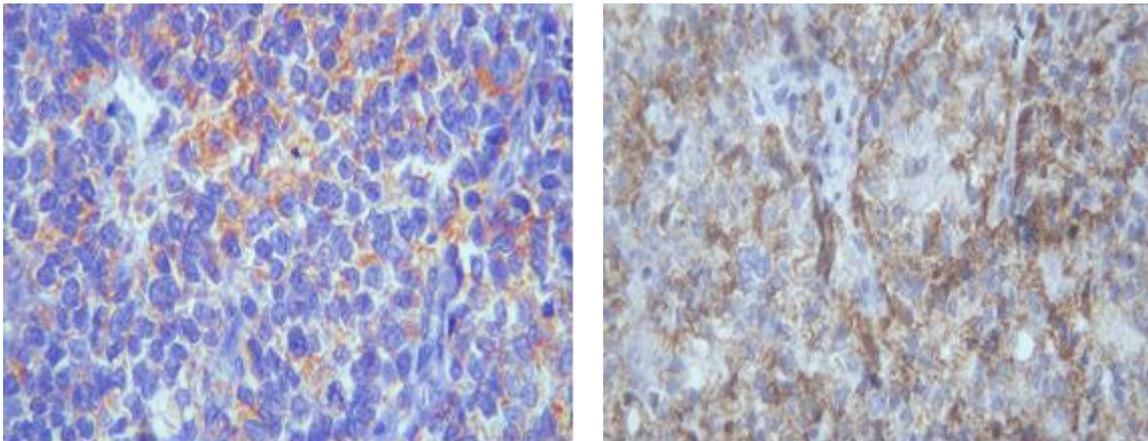
Fig. 4. A case of invasive ductal carcinoma GII with positive membranous-cytoplasmic immunostaining (Score III)

There was a weak positive correlation between HCMV viral proteins (E/IE) and COX-2 in group IA with statistically insignificant difference r= 0.26 and (p= 0.15) as shown in **figure 5.**
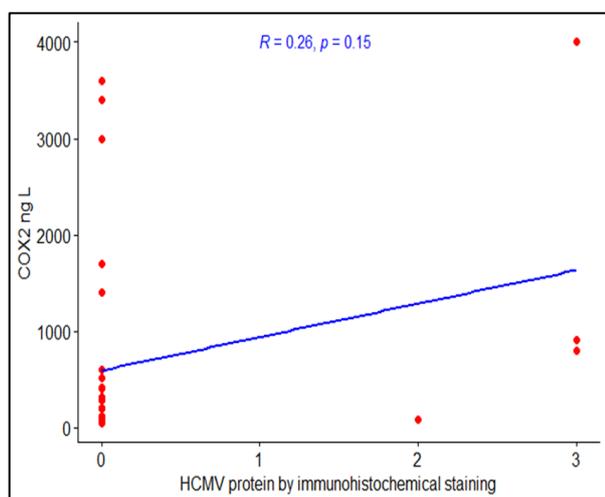
Fig. 5: Correlation between HCMV viral proteins and COX-2 in group IA breast cancer tissues

DISCUSSION

The production of several HCMV gene products by tumor cells in breast cancer, particularly triple negative breast cancer (TNBC), suggests that breast epithelial cells may have acquired an oncogenic infection as a result of breast cancer. Because certain strains of HCMV have the capacity to transform tumor cells into malignant ones, they may be the source of these infections [17].

The majority of women with breast cancer are middle-aged and older, according to the American Cancer Society (ACS). Sixty-two is the median age of a breast cancer diagnosis. This indicates that women who are diagnosed with breast cancer are half as old as 62 or younger [18].

The age distribution of groups IA, IB, and II in our study showed significant statistical differences (p value < 0.001). The mean age of group IA, which consisted of breast cancer patients, ranged from 24 to 73 years old. According to a meta-analysis research by Azim et al. [19], 57% of Egyptian BC patients were premenopausal or perimenopausal at the time of diagnosis, with a mean age of 50.4 years. This was much younger than the Western counterparts. The younger age of breast cancer in Egypt Possibly the population pyramid distribution explains, while only 8–9% of women in the population are above 60, other emerging nations, like China, exhibit a comparable age distribution, with the average age at presentation being 53.

In group IB patients (patients with benign breast lesions, mainly fibroadenoma), the age ranged from 35 to 53 years old, with 44.88±5.29 as the mean age. This contradicts research done by Das et al. [20], who found fibroadenoma patients had an average age of 20 years. More study indicates that fibroadenoma mainly affects women in the 14–35 age range, while it can furthermore strike older women [21]. Simple fibroadenomas were usually smaller in size and usually occurred in people with a median age of 28.5 years. Conversely, advanced age individuals—whose median age was 47 years— often displayed complex fibroadenomata [22].

In the present study, the main bulk of cases (81.8%) had an invasive duct carcinoma (IDC). These were in line with the findings of Azim et al.[19], who reported that invasive duct carcinoma was estimated to account for 80% of cases, whereas invasive lobular carcinoma constituted 7%.

Compared to grades 1 and 2, cancer categorized as grade 3, or high grade, is growing faster and has a higher chance of spreading [23]. Grade II breast cancer accounted for 78.8% of the cases in our study, while grade III accounting for 18.2% and grade I for 3%. This outcome is consistent with a research done by Omar et al.[24], which documented that the percentages of tumors in grades II and III were 66.0% and 28.6%, respectively. According to this study, regardless of therapeutic advancements, a larger occurrence of advanced stages is linked to a lower survival rate [19]. This highlights the critical role that early detection plays in improving the prognosis of disease in Egypt and other developing nations.

Human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR), and estrogen receptor (ER) positive were detected in 12.1%, 84.8%, 100% and of the cases in the current investigation, respectively. There were 39.4%, 21.2%, and 39.4% of cases with Ki-67 reactivity ranging from 0–16%, 16–30%, and > 30%, respectively. According to a comprehensive analysis of research on BC conducted in Egypt, the percentages of ER+ and PR+ patients were 70% and 61%, respectively, while the projected percentage of HER2+ subtype was 21% [19]. The development and tumor cells proliferation are substantially associated with Ki-67 expression [25].

With respect to group IB, 87.5% of cases had fibroadenoma identified, 8.3% had lipoma, and 4.2% had phyllodes tumor among all benign breast diseases. Fibroadenomas are the most common benign breast tumor, affect 25% of women [26].

Reactivation of the latent state depends on specific gene subsets that regulate HCMV replication, such as immediate early genes (IEI) [27]. Nested-type PCR was used in the current study for the immediate early (IE) gene detection in samples of breast tissue. In group IA, four samples (12%) tested positive for this gene; in groups II and IB, no detection of this gene was found. The three distinct groups (Groups IA, IB, and II) and the

HCMV IE gene showed a statistically significant weak correlation ($p = 0.036$ and $V = 0.283$).

Our study's findings regarding the detection of CMV DNA in breast cancer tissue are consistent with data reported by Mohammed et al. [28], who discovered that 23.7% of the evaluated breast cancer tissue samples tested positive for CMV using PCR. Additionally, our findings align with a study conducted by Elshazly et al. [29], which discovered that the positivity rate of HCMV in cancerous breast tissue was 18%.

Furthermore, it was shown by Eghbali et al. [30] that 8.3% of samples of breast cancer had positive HCMV PCR results. This is consistent with a research by Taher et al.[31], which reported that 12/12 (100%) of breast cancer specimens had CMV DNA. Earlier research by Tsai et al. [32-33], which shown a strong correlation between CMV and human breast cancer, is validated by these findings. In contrast, HCMV was not found in the examined breast cancer samples using PCR, according to the research done by Antonsson et al.[34] and Richardson et al.[35].

Identification of viral carcinogenesis, in which a virus infects cells and produces malignant transformation, but the viral genome is lost and cannot be recognized in cancer tissue, may be more challenging due to the possibility of viral "hit and run oncogenesis". An epigenetic signature left by the tumor virus has been suggested as a potential marker for this process[36]. This notion might be related to sample results showing negative levels of HCMV DNA. Furthermore, it's possible that incorrect storage caused the materials' negative PCR results because of a drop in viral nucleic acids or deterioration of viral genetic material[29].

Cyclooxygenase-2 (COX-2) is frequently expressed in different cancer cells and is associated with a bad prognosis. It's been shown that the prostaglandin E2 (PGE2) promotes tumor cell proliferation, division, and metastasis [14].

A statistically significant correlation was observed between the expression of COX-2 in ductal carcinoma in situ (DCIS) and invasive breast cancer in a study conducted by Leo et al. [37] and Harris et al. [38] revealed that COX-2 protein is present in all invasive human breast cancers but is absent from samples of normal breast tissue. This is in line with the results of our investigation, which revealed that the groups under investigation differed in their median COX-2 values in a statistically significant way, with group IA having a higher median value than groups II and B (p value = 0.0088). Nearby results were documented by Yoshimura et al.[39], who demonstrated that cells of breast cancer have far higher levels of immunoreactive COX-2 polypeptides than fibroadenoma cells. Fornetti et al.[40] however, reported that ovarian hormones were found to be modulators of COX-2 in normal mammary epithelium, especially at pregnancy levels.

In the current study, we identified CMV E/IE (viral replication) proteins in breast cancer (group IA) using immunohistochemistry. In cancerous cells, 75% of positive immunostaining for the CMV E/IE gene was cytoplasmic, while only 25% was nuclear. One explanation for the tested instances would be because they are at a later stage of infection of HCMV. This conclusion is in line with Elshazly et al.'s findings [29].

Our results corroborate the findings of Mohammed et al. [28], who reported significantly high expression levels of CMV proteins, including the IE1, the late PP65 in IDC, and the safety margins ($p<0.005$). Our findings also revealed high levels of CMV E/IE protein expression in tissue samples from breast cancer. In a different study, Taher et al.[31] showed that 100% of the examined primary breast cancer tissue samples expressed the CMV protein.

In group IA, there was a weak positive correlation ($r=0.26$) and statistically insignificant difference (p value=0.15) between the HCMV viral proteins (E/IE) and COX-2. This study and the one by Costa et al. [12] are comparable studies shown a strong correlation between high levels of HCMV IE protein expression and widespread COX-2 protein expression in breast cancer tissue samples.

CONCLUSIONS

The virus is prevalent in BC tissues since CMV proteins and DNA are found there. However, the essential conditions were not fulfilled for HCMV and BC to be causally related. Our findings further suggest that COX-2 may play a role in the emergence of breast cancer. Our results show a weakly positive connection between the overexpression of COX-2 in human breast cancer and the expression of the HCMV (E/IE) protein.

Recommendation:
- To determine the precise involvement of HCMV in breast cancer and the connection between COX-2 production and action and HCMV replication, more research is required.
- Subsequent research attempts should to concentrate on evaluating the function of specific COX-2 inhibitors in the management and prophylaxis of human breast cancer.

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