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A cross-sectional study for patients with Cytomegalovirus and breast cancer in Baghdad

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ABSTRACT

Breast cancer is one of the most common cancers among women worldwide and is particularly prevalent in Iraq, accounting for 32% of female malignancies. This study, conducted in Baghdad between January and December 2021, investigated factors influencing breast cancer and its relationship with cytomegalovirus (CMV) among 66 women aged 20–89 years diagnosed clinically and radiologically. Blood samples tested using ELISA revealed CMV-IgM and CMV-IgG positivity. Results showed 67.56% of married women with breast cancer used contraceptive pills, compared to 32.43% of unmarried women for menstrual regulation, while 93.10% of unmarried patients did not use them. The highest prevalence occurred in women aged 40–49 years (43.58% married, 37.03% unmarried). Family history was present in 48.48% of cases, while 51.51% reported none. Feeding patterns showed 70.03% of married women depended on artificial feeding, 11.11% on mixed feeding, and 51.85% on natural breastfeeding. Weight distribution indicated 30.30% of patients weighed 71–80 kg and 24.24% weighed 81–90 kg, with the most common height range being 161–170 cm (63.63%). Left breast cancer was more common than right, affecting 47.64% of married and 52.38% of unmarried women. ELISA testing revealed 26.92% of married and 73.07% of unmarried women were positive for both anti-CMV IgG and IgM, while human cytomegalovirus DNA was detected in all samples, confirming widespread HCMV infection among Iraqi women with breast cancer.

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Introduction

Breast cancer ranks first among cancerous diseases in terms of incidence, according to the Iraqi Ministry of Health records, with an estimated rate of 36.1% of the total number of cases (Abdul-Khaliq et al., 2019). Out of every three women diagnosed with cancer, one woman is diagnosed with breast cancer. According to estimates by the Central Statistics Agency, the country's population is expected to rise from about 36 million in 2016 to about 51 million by 2030, which will raise the number of cancer cases to about 460,000, assuming environmental and treatment factors are proven. Environmental activists warn of the high levels of pollution in the rivers as a result of the direct disposal of polluting materials in them without treatment and which are reused, in addition to the pollution caused by weapons during the wars in Iraq since the eighties of the last century (Ali, 2012; Stoltenberg et al. 2020).

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Types of breast cancer, which develop in different parts of the breast include Ductal carcinoma which affects the ducts in the breast, also known as ductal carcinoma in situ. This type of cancer does not spread to the tissues but rather infects the ducts, as detected by X-ray, and symptoms do not appear in the form of lumps in the breast. The other type of breast cancer is invasive cancer of the breast, which is the most widely used type, affecting the tissues and cells that surround the breast, the tissue that lines around the breast ducts. Less common types include inflammatory breast cancer, invasive lobular carcinoma, and Paget's disease of the breast (Ginsburg et al., 2020; Mutebi et al., 2020; Velazquez et al., 2018).

Breast cancer risk factors such as age, history of the family, reproductive health history and menstruation, and the use of hormonal contraceptives for long periods, Menstrual or hormonal factors include early onset of menstruation (before the age of twelve) or amenorrhea, Late menstruation (after 55) and taking hormonal drugs to stop menstruation, including estrogen hormones and progesterone, races, Postmenopausal obesity, lack of physical activity, drinking alcohol and/or smoking and take birth control pills (Ilbawi & Velazquez-Berumen 2018; McCormack et al., 2020; Rositch et al., 2020). Some local and international studies have found that there is a relationship between breast cancer and infection with the CMV virus, but in different proportions. In contrast, other studies have found that there is no relationship between the virus and the development of breast cancer. Therefore, the objectives of the research are to determine the factors that influence breast cancer and to study the relationship between cytomegalovirus and breast cancer.

Materials and Methods

Patients and sampling: the research was carried out between January 2021 and December 2021 in Central Public Health Laboratory and Medical City Hospitals in Baghdad. Clinically diagnosed with breast cancer and by X-ray imaging as well as mammography, which shows abnormalities and tissue changes in the breast. According to ethical considerations and medical consent, blood samples were collected from Al-Alweiya Teaching Hospital. Sixty-six (66) female breast cancer patients with ages ranging from 20 to 89 years were given ten milliliters of blood. Venipuncture samples were taken and collected in gel tubes. To obtain serum, blood samples were centrifuged at 3000 rpm for five minutes. Before the test, the serum in each tube was separated into two halves and deposited in Eppendorf tubes for evidence of being tagged and stored at -20C°. According to ethical considerations and medical consent, blood samples were collected from Al-Alweiya Teaching hospital. Sixty-six (66) female

breast cancer patients with ages ranging from 20 to 89 years were given ten milliliters of blood. Venipuncture samples were taken and collected in gel tubes. To obtain serum, blood samples were centrifuged at 3000 rpm for five minutes. Before the test, the serum in each tube was separated into two halves and deposited in Eppendorf tubes for evidence of being tagged and stored at -20C°. The Body Mass Index (BMI) was calculated for all patients according to the equation (1) followed Weir and Jan (2024).

$$BMI= mass (Kg) / height (m)^2$$

Enzyme-linked immunosorbent assay (ELISA) protocol

Cytomegalovirus – IgM (CMV- IgM) kit and Cytomegalovirus - IgG (CMV- IgG) kit (Bioactive diagnostica/ Germany) were used. Before use, all reagents and sera were gently mixed and allowed to cool to room temperature (20-25°C). The strips of microtitration were labelled in the same way as the samples of serum. Samples of sera were diluted with a sample diluent solution and completely homogenized. Wells were filled with prepared standards (cut-off, negative, and positive) and diluted sera.

Using a heating block microelisa system, for 30 minutes, the microtitration strips were covered and incubated at 37°C. Following incubation, the wells had to be aspirated and cleaned four times with washing solution for thirty seconds each time utilizing a Microplate washer with an automated system. By inverting the plate on absorbent material, the microtiter was then dried. 100 microliters of HCMV-HRP conjugate were applied to each well using a multichannel micropipette. Using a heating block microelisa system, the plate was incubated for 30 minutes at 37°C using the washing solution, the wells were aspirated four times and cleaned for 30 seconds each time. for each stage using an automatic microplate washer after the incubation period was over. By flipping the plate onto an absorbent medium, the micrometer was dried. Then, using the multichannel micropipette, 100 microliters of TMB chromogen solution (3,3', 5,5; - Tetramethylbenzidine) were added to each well-off light.

At 25°C, the plate was incubated in the dark for 15 minutes. Then 100 μl of stop solution was put in each well, the color changing from blue to yellow. Using a microplate reader (HumaReader Hs), the absorbance of each well was measured at 450 nm in under 30 minutes.

Deparaffinization and DNA extraction

The deparaffinization process was carried out using ethanol and xylene. All of the specimens were first put into microtubes, after which xylene was added and the tubes were centrifuged for one minute at 14,000 rpm and maintained at 45°C for fifteen minutes. This phase was

repeated. After discarding the supernatant, one milliliter of 100% ethanol was added to the precipitate. After ten minutes of storage at ambient temperature, it was centrifuged once more for one minute at 14,000 rpm. They threw away the supernatant. 70% ethanol was added, and the identical conditions were again applied to repeat this process. The pellet was used for DNA extraction after the supernatant was disposed of and all microtubes were heated to 65°C for five minutes in order to evaporate the ethanol residue (Habibian et al., 2013). DNA was extracted using a High Pure PCR Template Preparation Kit (Roche, Germany, code No: 11796828001) in accordance with the manufacturer's instructions. In preparation for PCR amplification, the isolated DNA was kept at -70°C.

Conventional PCR

To identify HCMV, the following primers were used: F primer 5́- TCTGGGAAGCCTCGGAACG-3 and R primer 5-GAAACGCGCGGCAATCGG-3. These primers were conserved for the GB region of HCMV. Ten microliters of extracted DNA, 2.5 microliters of PCR buffer 10X (Roche), 0.5 microliters of dNTP 10mM (Roche), 1U Taq Polymerase (Roche), and 1 microliter (20µM) of each primer sequence were used in the first round of PCR, which was conducted in a 25-microliter mixture. The mixture was then placed in a thermocycler (Techne TC-5000, UK) (Gilbert et al., 1999). The 581 bp items show a positive reaction.

Gel Electrophoresis

After being separated on a 2% agarose gel, the PCR product was produced using Safe Stain at a voltage of 100V. The outcome was observed in a transilluminator under UV light. A 100 bp ladder, which was positioned on the well as an indicator, was used to compare the band sizes.

Statistical analysis

The prevalence rates of breast cancer and anti-CMV positive were compared using Pearson's chi-square (X2) test and Fisher's exact probability. The significance level was set at p = 0.05.

Results

The current study's results revealed that the number of Iraqi women diagnosed with cancer is constantly increasing. It was found that 25 (67.56%) of the infected married women take contraceptive pills, while 12 (32.43%) of the infected unmarried women take oral contraceptive pills to regulate their menstrual cycle. Also, it was found that 27 (93.10%) of the infected unmarried women do not take oral contraceptive pills (Table 1). The findings revealed that the highest prevalence of breast cancer was within the age group 40-49 years for married and unmarried women, 17 (43.58%) and 10 (37.03%), respectively (Table 1).

There are no significant differences in the family history of women with and without breast cancer. 32 (48.48%) of the women had a breast cancer family history on either the mother's or father's side or both. There was no family history of cancer in 34 (51.51 percent) of women diagnosed with breast cancer (Table 2). It was found that 10 (70.03%) of married women diagnosed with breast cancer depend on artificial feeding to feed their children after childbirth, 3 (11.11%) rely on both artificial and natural breastfeeding even though they have little milk in their breast or because they are employed and work long hours outside the home. In comparison, 14 (51.85%) of the infected women depended on breastfeeding and feeding their children (Table 2).

Table 1 Demographic characteristics of the patients included in the study

Women	Women taking contraceptive pills (%)	Women do not take contraceptive pills (%)	Total (%)	Probability
Married (%)	25 (67.56)	2 (6.89)	27 (40.90)	6.5 x 10⁻⁷
Single (%)	12 (32.43)	27 (93.10)	39 (59.09)	
Total (%)	37 (100)	29 (100)	66 (100)	
Age (year)	**Married (%)**	**Single (%)**	**Total (%)**	
20 - 29	1 (3.70)	0 (0)	1(1.51)	
30 – 39	3 (11.11)	8 (20.51)	11(16.66)	
40 – 49	10 (37.03)	17(43.58)	27(40.90)	
50 - 59	6 (22.22)	7(17.94)	13(19.69)	
60 - 69	6 (22.22)	6 (15.39)	12(18.18)	
70 - 79	0 (0)	1(2.56)	1(1.51)	
80 - 89	1 (3.70)	0(0)	1(51)	
Total (%)	**27 (100)**	**39(100)**	**66(100)**	
Artificial feeding (%)	**Natural and artificial feeding(%)**	**Natural feeding (%)**	**Total (%)**	**Probability**
10 (70.03)	3 (11.11)	14 (51.85)	27 (100)	5.7 x 10⁻³

Table 2 Family history of women with breast cancer and the type of breastfeeding used by married women

Family history		Total (%)	Probability
Positive (%)	Negative (%)		
32 (48.48)	34 (51.51)	66 (100)	0.728
Artificial feeding (%)	Natural and artificial feeding (%)	Natural feeding (%)	
10 (70.03)	3 (11.11)	14 (51.85)	5.7×10^{-3}

Most of the infected women were suffering from a relative increase in weight, as it was found that women who weighed (71-80) kg, followed by those who weighed (81-90) kg, with a percentage of 20 (30.30%) and 16 (24.24%) respectively are the highest percentage of weights. Also, their prevalent heights were 161-170 cm, with a percentage of 41 (62.12 %). Most of patients had a BMI between normal and obese class II (Table 3). The highest occurrence of left breast cancer among married and unmarried women was higher than the right breast, as the incidence of left breast cancer for married women was 20 (47.64%) and for single women 22 (52.38%). As for the right breast cancer incidence among unmarried women, the incidence rate of single women was 17 (70.83%) and 7 married women (29.16%) (Table 4).

Table 3 Weight and height of women with breast cancer.

Weight (Kg)	No. of patients (%)	Height of patients (cm)	No. of patients (%)
41-50	5 (7.57)	131-140	1 (1.51)
51-60	6 (9.09)	141-150	1 (1.51)
61-70	10 (15.15)	151-160	20 (30.30)
71-80	20 (30.30)	161-170	42 (63.63)
81-90	16 (24.24)	171-180	2 (3.03)
91-100	6 (9.09)		
101-110	3 (4.54)		
Total (%)	**66 (100)**		**66 (100)**

Table 4 Percentages of left and right breast cancer among married and unmarried women.

Status	Left Breast (%)	Right Breast (%)	Total (%)	Probability
Married (%)	20 (47.61)	7 (29.16)	27 (40.90)	0.142
Single (%)	22 (52.38)	17 (70.83)	39 (59.09)	
Total (%)	42 (100)	24 (100)	66 (100)	

The anti-IgG and IgM enzyme-linked immunosorbent assay were used on a total of 66 sera of patients with breast cancer diagnosed clinically using mammography and X-rays. The results showed that 7 (26.92%) of the married women gave positive results for the anti-CMV IgG and anti-CMV IgM test, while 19 (73.07%) were unmarried (Table 5). The GB region of HCMV was present in all samples of patients that were detected using PCR (Figure 1).

Table 5 Anti-CMV IgG and anti-CMV IgM test among married and unmarried women in this study.

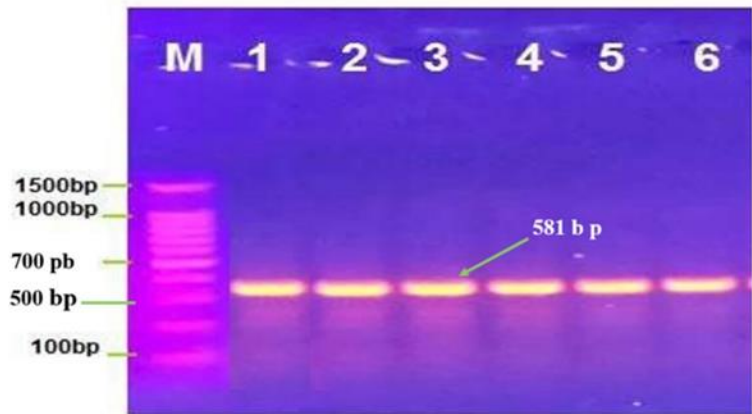| Status	Anti-CMV IgG and anti-CMV IgM (+ve) (%)	Anti-CMV IgM and anti-CMV IgG (-ve) (%)	Total (%)	Probability
Married (%)	7 (26.92)	20 (50)	27 (40.90)	0.062
Single (%)	19 (73.07)	20 (50)	39 (59.09)	
Total	26 (100)	40 (100)	66 (100)	
Tumor grade II	7/26 (26.9)	25/40 (62.5)	66(100)	0.05
Tumor grade III	19/26 (73.1)	15/40 (37.5)	66(100)	
Mastitis invasion	18/26 (69.23)	14/40 (35)	66(100)	
Local (In situ)	8/26 (30.76)	26/40 (65)	66(100)	

Fig 1. Agarose gel electrophoresis showing amplified products (581 bp). Lane M: 100 bp DNA ladder; Lanes 1–6: PCR-amplified samples.

Discussion

The present study's findings are in line with previous findings, local and international studies. Where the results of the study by researchers Mohammed et al. in 2020 showed that the highest incidence of cancer was breast cancer, with a rate of 54.67%, particularly in the 40-49-year-old age group, with a percentage of (35.52%) (Mohammed et al., 2020). In a similar study, researchers found that Anti-HCMV IgG Ab appeared in 90% of healthy women, 95% of benign breast cancer patients, and 100% of malignant breast cancer patients (Bareq et al., 2018). In China, a cross-sectional study has enlisted the help of 274 women. Awareness of breast cancer was moderate (median = 76.50) among participants.

It was positively connected with a high level of literacy in health information, educated spouses, consulting a doctor after noticing suspicious breast changes and living close to a hospital (Na et al., 2020). Gene products of the human cytomegalovirus (HCMV) have been detected in >90 percent of breast cancer tumors and metastases, according to new research, and their greater expression has been linked to a more aggressive breast cancer phenotype. By directing Immunosuppressive type II macrophage polarization, blocking antigen presentation, producing T-cell inhibitory molecules, and possibly inducing regulatory T-cell reactivity, infected cells with HCMV can resist identification and destruction via means of the immunological system (Jürgen et al., 2019).

Another study discovered that 20 of 37 individuals with ductal breast cancer (54.04 percent) tested positive for HCMV DNA after diagnosis of cases using the polymerase chain reaction (PCR) technique to diagnose cases. In the age categories 30-39, 40-49, and >50 years old, the prevalence of HCMV DNA was also high: 7 (72.22 percent), 9 (69.23 percent), and 4 (57.14 percent), respectively (Peyman et al., 2019).

Even though there are variances in the results of earlier researchers who published epidemiological and laboratory investigations, this could be due to differences in blood sample collection time or the high rate of CMV seroprevalence; sample size, age of the sample, and diagnosis procedure (Richardson et al., 2020). 14 (1.9%) of the 723 Iraqi women evaluated had a family history of

breast cancer, while 22 (3%) had current or prior breast illnesses that required medical attention. A mammogram is something that more than half of women (61.2%) had heard of (Taqi et al., 2021). In other studies, two hundred and fifty eight women, ranging in age from 20 to 79, were diagnosed with breast cancer. The diseases were most prevalent in the fourth and fifth decades of life. Single people made up about 10% of the population by sector of residence. Contraceptives and hormone therapy were used by 42% of the women, and 15% had previously experienced breast cancer. Non-lactating women make up 24% of the population, whereas smokers make up 6% (Rasim et al., 2015). In Basrah, 61.7 % of the 300 women who took part in the study were married. 16.7% of women had a history of breast cancer in the family, according to the study. Mammography was shown to have indicated their ailment in 38.3 % of patients (Aliyah et al., 2020).

In 2017, a cross-sectional study was done in Babylon and Karbala in Iraq. Ultrasonography and elastic stress imaging were used to examine clinical samples of women who had a cancerous breast mass. They were between the ages of 16 and 69 years. With a sensitivity and specificity of 93.5 % and 100 %, ultrasound properly diagnosed 58 of the 62 malignant breast tumors (Almosawi et al., 2017). In another study, the mean strain ratio of metastasis-affected lymph nodes was larger than that of reactive lymph nodes, 5.04 ±1.96 versus 1.7 ± 0.87, with a highly significant difference (Mohammed et al., 2017).

A total of 250 breast cancer patients' clinical samples were tested at random. Breast cancer was prevalent among 50 to 59-year-old women. Married females made up a major fraction of the study population, which is consistent with current, national, and international breast cancer prevalence data (Al-Fadhli et al., 2016). Iraqi researchers and doctors have also found that a 23% mortality rate is related to breast cancer (AL-Behadily & Al-Tukmagi 2017).

Almusawi and Kadhim in 2021 were found a sensitivity of 100 % and a specificity of 34.6 %, 21 of the 47 lesions were determined to be malignant (by fine-needle aspiration (FNA) in 19 lesions and excisional biopsy or mastectomy in 28), giving a sensitivity of 100 % and a specificity of 34.6 % (Almusawi & Kadhim, 2021). While the results of another study showed that individuals in the 40-49 year and 50-59 year decades of life have the highest incidence of breast cancer (32 %), with estrogen and progesterone levels (74 % and 75 %, respectively) and 69 patients (69 %) (Zainab & Kadhim, 2021).

Mohamed et al. (2019) found that there were 176 non-pregnant patients (57.51%) and 130 pregnant patients (42.48%) in the non-pregnant group, and the primary age group with the most cancer was 30-39 years. There were 62 patients (20.3%) who had a history of breast cancer in their family, while 244 patients (79.7%) had no history of breast cancer in their family. 258 patients (84.3%) had regular periods, whereas 48 patients (15.7%) had irregular periods. 96 patients (31.4%) had utilized hormone therapy in the form of tablets or injections, 258 patients (84.3%) had regular periods, while 48 patients (15.7%) had irregular periods. Another 80 patients (26.1%) utilized formula, while 226 patients (73.9%) were breastfeeding their children. Fibrous adenoma was identified in 46 patients, mostly in the 20–29-year-old age group (39 %) (Mohamed et al., 2019).

Evidence suggests that the proteins US28, pp65, and IE1, which are encoded by HCMV genes during the latent infection phase, may accelerate the growth of some cancers, despite the fact that the link between HCMV and cancer is debatable. HCMV DNA is highly prevalent (54.05%) in ductal cancer patients (17). The Egyptian researchers El-Shinawi et al. (2013) similarly found a strong correlation between HCMV and breast cancer, with 82% of 28 patients with inflammatory breast cancer having greater serum levels of HCMV IgG than 49 individuals with non-inflammatory breast cancer (El-Shinawi et al., 2013). In a different investigation, HCMV DNA was found in fewer samples (13, 19%) from aborted women than from pregnant women (three, 15%) (AL-Hajjar & Al-Mousawi, 2021).

Conflict of interest
The authors declare that they have no conflict of interest.

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