Original Article

Investigation the role of human cytomegalovirus in the invasive ductal breast carcinoma

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ABSTRACT

Background: Breast cancer is the most common type of female malignancy. Increasing evidence in the last 10 years suggests that human cytomegalovirus (HCMV) is associated with several human malignancies including breast cancer. This study aimed to investigate HCMV in invasive ductal breast carcinoma by enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry (IHC). **Materials and Methods:** A total of 50 samples of the cancer mass and non-neoplastic safe margin (SM) tissues of breast cancer collected then processed for paraffin block to apply IHC and hematoxylin-eosin staining. In addition to that, 30 blood samples collected from patients and healthy women (controls) for detection of anticytomegalovirus (antiCMV) IgG and IgM by ELISA. **Results:** About 38 samples (76%) of 50 samples diagnosed as invasive ductal carcinoma (IDC). The results showed that the presence of antiCMV antibody IgG in 100% of patients while the IgM presented in 76.7% of patients. There were significant differences ($P < 0.05$) between the optical densities of the IgG in breast cancer patients when compared healthy women. The positive results of CMV protein comprise 34 (89.4%) for immediate early 1 (IE1) protein, 35 (92.1%) for late protein, and 34 (89.4%) for phosphoprotein 65 (pp65) from 38 sample of IDC. The results also showed the absence of expression to CMV late and pp65 proteins and low percentage (10%) of IE1 protein in the SM tissues. **Conclusion:** Many studies including our observation indicated to the association of HCMV with breast cancer, but the role of HCMV in the pathogenesis of breast cancer is unclear.

Key words: Breast cancer, cytomegalovirus, enzyme-linked immunosorbent assay, immunohistochemistry

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer in women worldwide with an estimated 1.4 million new cases in 2008.[1] In Iraq, breast cancer is the most common type of female malignancy, accounting for approximately one-third of the registered female cancers according to the 2008 Iraqi Cancer Registry.[2] This shows that the breast is the leading cancer site among the Iraqi population in general, surpassing even bronchogenic cancer.[3]

As in all cancers, the cause of breast cancer remains unknown. Research into its etiology has focused primarily on reproductive and other factors affecting circulating sex hormones as well as on genetic susceptibility. Hormones, as identified risk factors thought to explain only about half of all breast cancer incidences. Researchers are motivated to consider other routes of disease pathogenesis.[3]

The International Agency for Research on Cancer reports that biological carcinogens cause 18-20% of cancers, suggesting the tremendous potential of controlling microbe-related processes for cancer prevention.[4]

Human herpes viruses are known for its oncogenic potential. Human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) of the herpesviridae family have been implicated as a cause of breast cancer. There was significance of EBV expression in breast tissue of young patients with breast cancer[5] and this finding confirmed by polymerase chain reaction-based studies, positive correlation was shown,[6] and the presence of EBV DNA was associated with more severe forms of breast cancer.[7]

Human cytomegalovirus had be shown to be involved in many cancers including malignant glioma, and prostate, skin, and colorectal cancers.[8]

Access this article online

Quick Response Code:

Website: www.ccij-online.org

DOI: 10.4103/2278-0513.148915

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Increasing evidence in the last 10 years suggests that HCMV is associated with several human malignancies and that HCMV gene products can modulate oncogenic properties of cells *in vitro*.[9] Since persistent of HCMV infection of breast epithelium could, in theory, promote malignant transformation of infected breast epithelium that sought to determine the HCMV gene products in normal and neoplastic breast.[10]

There might be several mechanisms of how cytomegalovirus (CMV) can cause breast cancer initiation and progression. First, it was shown that HCMV gene products affect cell cycle regulation, inhibit apoptosis, activate angiogenesis, and metastatic phenotype, and cause increased mutation rate, thereby overlapping with all established hallmarks of cancer cells.[11]

Second, HCMV exhibits immunosuppressive properties, leading to escape of tumor cells from immune surveillance mechanisms.[12] HCMV has evolved multiple strategies for immune evasion resulting in persistent viral infection in the host.[13] Several HCMV proteins, including those expressed with immediate early (IE) genes, block the host cell major histocompatibility complex (MHC) class I antigen expression, which is essential for activation of CD 8+ T-lymphocyte antitumor cytotoxicity. HCMV UL83 protein (phosphoprotein 65 [pp65]) blocks antigen presentation of HCMV epitopes to CD +8 T-cells, and expression of HCMV UL18, a MHC class I homologue, disrupts "natural killer" cell recognition of HCMV-infected cells.[14]

Third, specific actions of virus-encoded interleukins (IL) could be implicated. IL-10 was shown to be differentially expressed in breast tumor cells and infiltrating lymphocytes. Elevated serum level of IL-10 observed in breast cancer patients. The fact that HCMV expresses a viral analog of human IL-10 may lead to the conclusion that this could be one of the mechanisms of breast cancer promotion by the virus.[15]

MATERIALS AND METHODS

Histopathology

The study prospectively designed. A total of 50 samples of the cancer mass and safe margin (SM) tissues of breast cancer collected under supervision of surgeons from Al-Imamin Al-Kadhimin Teaching Hospital and Dijlah Private Hospital during February–July 2013. In addition to collect 30 blood samples from the same patients and 30 blood samples from healthy women as controls.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits from HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Max-Planck-Ring21. 65205 Wiesbaden. Germany used for the detection of IgM/IgG antibodies to CMV in human serum. Samples prepared by diluted 10 µl of patient's serum added to 1 ml of dilution buffer IgM/IgG. Then, incubated for 5 min prior to further processing, 100 µl of negative control (NC) in duplicate, positive control (PC) in duplicate and diluted sample were pipetted to the coated wells, and then the microtiter plate covered with adhesive strips and incubated for 30 min at 17-25°C. Automated washing achieved for 4 times with 350 µl of working washing buffer. Then, 100 µl of anti-IgM/IgG conjugate added to the reaction's wells and incubated for 30 min at 17-25°C. Washing repeated, and 100 µl of substrate solution added to the reaction's wells and incubated for 15 min at 17-25°C. Finally, 100 µl of stop solution added, and the absorbance measured at 450 nm by Biotek L×800 Absorbance Instrument, 100 Tigan Street, Winoski, VT 05404, USA.

Cutoff value (COV) = Mean of NC + (0.2* mean PC)

Interpretation of results

- Absorbance at 450 nm (patient) ≥ COV + 15%: Consider antiCMV IgM/IgG antibody positive
- Absorbance at 450 nm (patient) < COV – 15%: Consider antiCMV IgM/IgG antibody negative.

Immunohistochemistry reaction

Paraffin blocks sectioned into 5 µm thick sections, using manual microtome. From each tissue block, serial sections collected. Those sections that mounted on positive charged slides used for Immunohistochemistry (IHC) procedure, detecting HCMV IE1 protein, late antigen and pp65 all these monoclonal antibodies and Secondary Detection Kit from Abcam plc, 330 Cambridge Science Park Cambridge CB4 0FL UK. One of the sections mounted on ordinary slides and used for hematoxylin and eosin stains. The procedure of the IHC assay adopted by this study was according to the staining protocol supplied with the detection kit.

All tissue sections were de-paraffinized then cleaned with xylene and descending concentration of ethyl alcohol. Tris-ethylenediaminetetraacetic acid buffer pH 9.0 used for antigen retrieval step then hydrogen peroxide blocking and protein blocking performed. The primary antibody applied, and the slides incubated for 1 h at 25°C. After washing, Mouse Specifying Reagent unconjugated (secondary antibody) added, and the slides incubated for 30 min at room temperature. Then washing and goat antirabbit horseradish peroxidase conjugate applied. Diaminobenzidine added, hematoxylin staining achieved, and finally, the slides mounted with distrene plasticizer xylene and covered with coved slip.

Ethical approval

This research underwent to the terms of ethical considerations and in accordance with the form prepared for this purpose

by the Iraqi Ministry of Health also got the approval of the research by the Committee of ethical standards in the Faculty of Medicine, Al-Nahrain University, one of the colleges affiliated to the Ministry of Higher Education and Scientific Research, Iraq.

RESULTS

Results of histopathology

About 38 samples (76%) of 50 samples had been diagnosed as invasive ductal breast carcinoma [Figure 1], 28 samples (73.7%) of 38 were estimated as grade 2 while 10 samples (26.3%) was grade 1 [Figure 2]. Pathologist did this diagnosis after hematoxylin and eosin staining achieved.

Results of enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay of antiHCMV antibody of two classes IgG and IgM performed on 30 patient's serum with invasive ductal carcinoma (IDC), in addition to 30 healthy women's serum as NC, Tables 1 and 2 display these results in detail. The results showed the presence of antiCMV antibody IgG in 100% of patients while the IgM presented in 76.7% of patients.

Regarding to the NCs (healthy women), the percentage of positive results showed almost the same as the patient's results, where antiCMV IgG present in 100% of healthy women while antiCMV IgM present in 50%.

The results showed no significant difference in the detection of antiHCMV antibody IgG between patients and NCs ($P > 0.05$) while there was significant difference ($P < 0.05$) in the detection of antiHCMV IgM in the patients when compared with NCs and the relative risk was 1.35 indicated that HCMV infection was more likely in patients than NCs.

The optical density (OD) of antiHCMV IgG antibody was higher in the patients group [Figure 3] and the results showed significant difference ($P < 0.05$) between the optical densities of antiHCMV IgG antibody in breast cancer patients when compared with NCs.

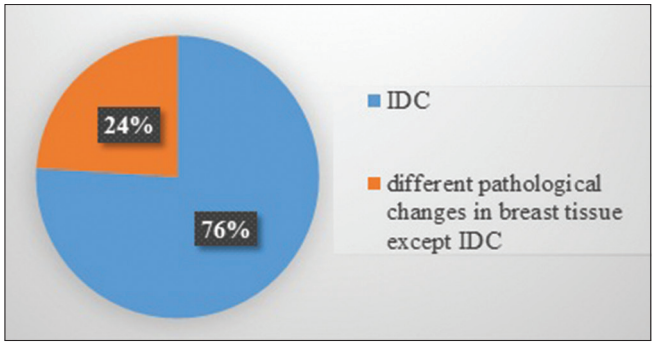

Figure 1: The percentage of invasive ductal breast carcinoma among different pathological changes in breast tissue

Results of immunohistochemistry

The expression of CMV proteins in invasive ductal breast carcinoma and nonneoplastic SM tissue samples was observed by IHC reaction. Tables 3-5 display the result of immunohistochemical reaction with statistical analysis for these results. The results showed significant differences ($P < 0.005$) in the expression of CMV proteins including IE1 [Figure 4], late [Figure 5], and pp65 [Figure 6] in the invasive ductal breast carcinoma tissue samples when compared with nonneoplastic SM tissue samples.

Table 1: Serological detection of antiCMV antibody IgG in women with IDC and negative controls

IgG	Study groups		Total
	IDC	Negative controls	
Positive			
Count	30	30	60
Percentage	100.0	100.0	100.0
Total			
Count	30	30	60
Percentage	100.0	100.0	100.0
Mean of optical density	1.803	1.161	

IDC: Invasive ductal carcinoma, CMV: Cytomegalovirus, IgG: Immunoglobulin G

Table 2: Serological detection of antiCMV antibody IgM in women with IDC and negative controls

IgM	Study groups		Total
	IDC	Negative controls	
Positive			
Count	23	15	38
Percentage	76.7	50.0	63.3
Negative			
Count	7	15	22
Percentage	23.3	50.0	36.7
Total			
Count	30	30	60
Negative	100.0	100.0	100.0
P	0.030		
Relative risk with (CI)	1.53 (1.02-2.3)		

CI: Confidence interval, IDC: Invasive ductal carcinoma, CMV: Cytomegalovirus

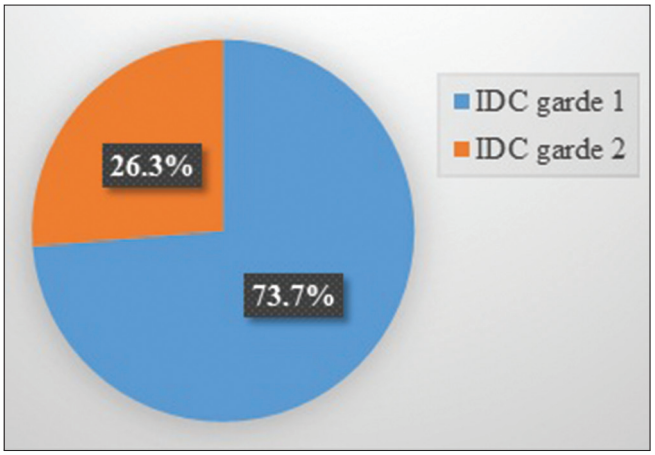

Figure 2: The percentage of invasive ductal breast carcinoma according to the grade of differentiation

The relative risk value indicates to strong evidence of an association between HCMV proteins and invasive ductal breast carcinoma. The positive results of CMV protein comprise 34 (89.4%) for IE1 protein, 35 (92.1%) for late protein, and 34 (89.4%) for pp65 from 38 sample of invasive ductal breast carcinoma. The results also showed the absence of expression to CMV late and pp65 proteins and low percentage (10%) of IE1 protein in the nonneoplastic SM tissues.

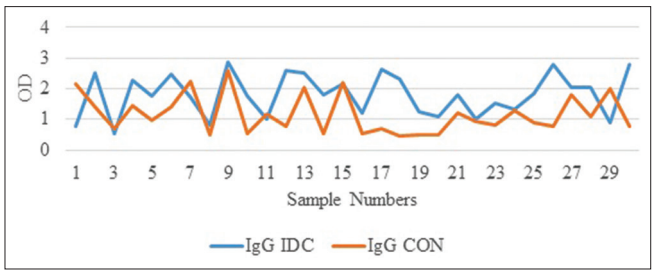

Figure 3: Optical density of antihuman cytomegalovirus IgG antibody for patients of invasive ductal carcinoma and negative controls

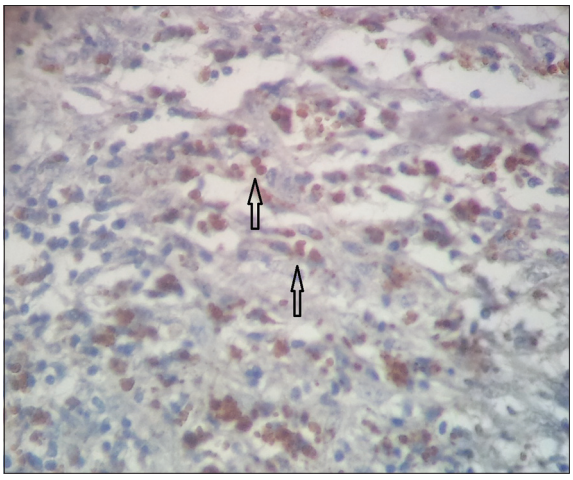

Figure 4: Immunohistochemical expression of human cytomegalovirus immediate early 1 protein in invasive ductal carcinoma showing positive brown nuclear staining as indicated by arrow (×40)

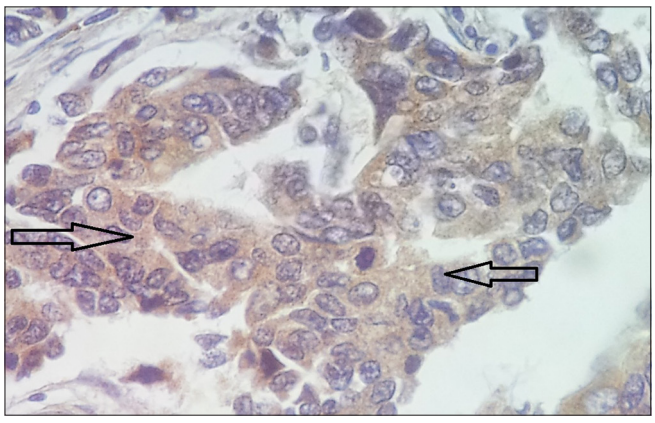

Figure 5: Immunohistochemical expression of human cytomegalovirus phosphoprotein 65 in invasive ductal carcinoma showing positive brown cytoplasmic staining as indicated by arrow (×40)

There were no significant differences regarding the expression of the HCMV proteins (IE1, late, and pp65) and the grade of invasive ductal breast carcinoma tissue [Table 6]. However, the results showed a high percentage of the expression of HCMV proteins in the grade 2 more than

Table 3: Immunohistochemical expression of HCMV IE1 protein in IDC and nonneoplastic SM tissue samples

IE1	Study groups		Total
	IDC	SM	
Positive			
Count	34	3	37
Percentage	89.5	10.0	54.4
Negative			
Count	4	27	31
Percentage	10.5	90.0	45.6
Total			
Count	38	30	68
Percentage	100.0	100.0	100.0
P	0.001		
Relative risk with (CI)	8.9 (3.04-26.33)		

IE1: Immediate early 1, HCMV: Human cytomegalovirus, IDC: Invasive ductal carcinoma, SM: Safe margin, CI: Confidence interval

Table 4: Immunohistochemical expression of HCMV late protein in IDC and nonneoplastic SM tissue samples

Late	Study groups		Total
	IDC	SM	
Positive			
Count	35	0	35
Percentage	92.1	0.0	51.5
Negative			
Count	3	30	33
Percentage	7.9	100.0	48.5
Total			
Count	38	30	68
Percentage	100.0	100.0	100.0
P	0.001		
Relative risk with (CI)	56.35 (3.6-883.84)		

HCMV: Human cytomegalovirus, IDC: Invasive ductal carcinoma, SM: Safe margin, CI: Confidence interval

Table 5: Immunohistochemical expression of HCMV pp65 in IDC and nonneoplastic SM tissue samples

Pp65	Study groups		Total
	IDC	SM	
Positive			
Count	34	0	34
Percentage	89.5	0.0	50.0
Negative			
Count	4	30	34
Percentage	10.5	100.0	50.0
Total			
Count	38	30	68
Percentage	100.0	100.0	100.0
P	0.001		
Relative risk with (CI)	54.84 (3.5-859.44)		

HCMV: Human cytomegalovirus, IDC: Invasive ductal carcinoma, SM: Safe margin, CI: Confidence interval, pp65: Phosphoprotein 65

Table 6: The expression of cytomegalovirus proteins according to the grade of differentiation of the IDC tissue

Histopathological grade of IDC tissue	IDC grade 1 (%)			IDC grade 2 (%)		
HCMV proteins	IE1	Late	Pp65	IE1	Late	Pp65
Positive	9 (23.7)	9 (23.7)	9 (23.7)	25 (65.8)	26 (68.4)	25 (65.8)
Negative	1 (2.6)	1 (2.6)	1 (2.6)	3 (7.9)	2 (5.2)	3 (7.9)
Total	10 (26.3)	10 (26.3)	10 (26.3)	28 (73.7)	28 (73.7)	28 (73.7)

HCMV: Human cytomegalovirus, IDC: Invasive ductal carcinoma, IE1: Immediate early 1, pp65: Phosphoprotein 65

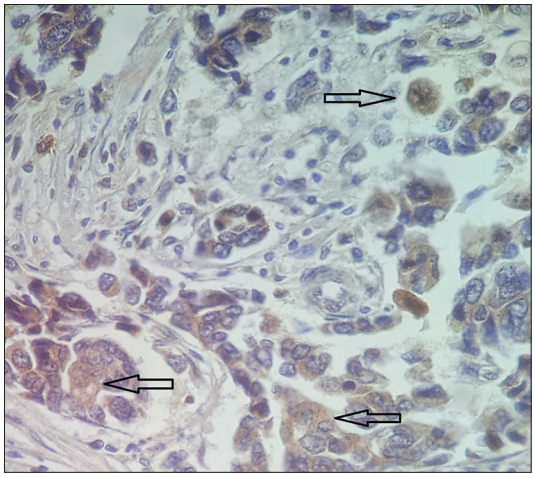

Figure 6: Immunohistochemical expression of human cytomegalovirus late proteins in invasive ductal carcinoma showing positive brown cytoplasmic staining as indicated by arrow (×40)

grade 1 of IDC tissue sample. In grade 1, the CMV proteins including IE1, late and pp65 showed 9 (23.7%) positive results out of 38 of IDC sample while in grade 2, the results observed as 25 (65.8%) for IE1 and pp56 and 26 (68.4%) for late protein.

DISCUSSION

Many studies indicated to the association of HCMV with breast cancer, but different results had been showed. The serological level considered screening test to diagnose the infection with HCMV in invasive ductal breast carcinoma and compared with healthy women (controls). Detection of antiHCMV IgG indicated to past or chronic infection while antiHCMV IgM indicated to acute or recurrent infection.[16] The result of this study showed 100% infection with HCMV in both IDC patients and controls and this result confirmed by Richrdson et al. study.[17] This result was not be a coincidence and the evidence to prove that was the significant difference in the OD of IgG between the IDC patients and controls. Cox et al.[10] study confirmed the significant difference in the OD of IgG between patients with breast cancer and controls, and supported that the seroconversion of CMV IgG and increasing IgG level were associated with an increased risk and precede the development of breast cancer among parous women. AntiHCMV IgM was used to prove which the higher HCMV IgG levels found in seropositive women with breast cancer could be the result of a more recent infection with HCMV.[18] The antiHCMV IgM positive results with a high level of IgG would be consistent with the hypothesis that late exposure to HCMV is a risk factor for breast cancer.[17]

Immunohistochemistry technique was used in this study to detect the proteins of HCMV in the IDC and nonneoplastic SM tissue samples. Three proteins of HCMV have been used, IE1 protein, late protein and phosphoprotein 65 (pp56). IE1 characterized by interaction and inactivation proteins of retinoblastoma (Rb) family promoting entry into S phase of the cell cycle.[19] Late HCMV proteins are expressed later in the virus life cycle and are mainly structural proteins forming the virus particles.[20] Pp65 is the major tegument protein responsible for modulating evading the host cell immune response during HCMV infections.[21]

The results of this study showed high expression of HCMV proteins in IDC tissue samples and significantly different from not neoplastic SM tissue samples. This finding confirmed by Taher *et al*.[20] study. Another study, Harkins *et al*.,[9] showed the expression of the HCMV proteins in both breast cancer tissue samples and normal epithelial cell of breast tissue but there was a significant difference in results between those samples. There were other studies[22,23] used IHC to detect HCMV proteins in malignant tissue (glioblastoma and colorectal cancer) and adjacent normal tissue. The results of these studies were identical to the result of this study, the expression of HCMV proteins was 80-100% in malignant tissue and absent from nonneoplastic SM tissue samples.

A major point of concern is that HCMV is not considered to be a tumor virus due to a lack of proven transformation potential in human cells. To explain the frequent presence of HCMV in tumor tissue, it proposed the concept of oncomodulation.[24]

Oncomodulation means that HCMV may infect tumor cells and increase their malignancy and it postulated that tumor cells provide a genetic environment characterized by disturbances in intracellular signaling pathways, transcription factors, and tumor suppressor proteins that enabled HCMV to exert its oncomodulatory potential while it cannot be manifested in normal cells. By this concept,

HCMV was proposed to be a therapeutic target in a fraction of cancer patients.[19]

Cytomegalovirus-infected cells exhibit increased cellular motility and invasion, reduced p53 and Rb function, elevated levels of telomerase, and increased resistance to chemotherapy-induced apoptosis.[18] This might suggest that HCMV positive cancer patients would benefit from anti-viral therapy combined with traditional chemotherapy.[25]

Chronic HCMV infection could potentially promote important oncogenic signaling pathways since HCMV infection expresses a chemokine receptor US28, which has oncogenic potential and has been shown to signal through the NF-kB pathway and activate downstream COX-2, STAT-3 and IL-6 expression.[26] At least four proteins encoded by the Unique Short region of HCMV genome have been involved in the inhibition of MHC class I expression, either by directly acting on MHC class I molecules or by acting on MHC class I associated proteins, including transporter associated with antigen processing and tapasin which have both chaperone-like and catalytic functions on MHC class I molecules.[27] HCMV and tumors considered two players for one goal that was an escape from the immune response. HCMV is a latent herpes virus that maintains dynamic relationships with the immune system and proposes that it has the potential to help the tumor cell to evade the first line of host defense and induce a state of immune tolerance in which it can grow.[28]

SUMMARY

Human cytomegalovirus is not oncogenic virus but exploits from some environmental changes occur in tumor cells in addition to ability of the virus like induction of inflammation that reflect in current study by detection of antiHCMV IgG and IgM in patients sera. The virus also have some genes and proteins such as UL36, UL37, and IE1 interfere with cell cycle proteins (p53 and Rb) leading to resistance of apoptosis and increase mitosis rate, the current study detected IE1 protein in IDC tissue sample and improve this fact.

ACKNOWLEDGMENT

This research funded by the Research and Development Department (RDD) of the Iraqi Ministry of Higher Education and Scientific Research, project of supporting postgraduate students (www.rddiraq.com).

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Cite this article as: Mohammed AH, Kadhim HS, Ghani AH. Investigation the role of human cytomegalovirus in the invasive ductal breast carcinoma. Clin Cancer Investig J 2015;4:199-205.

Source of Support: Nil, **Conflict of Interest:** None declared.