MOLECULAR DETECTION OF HUMAN CYTOMEGALOVIRUS IN IRAQI PATIENTS WITH BREAST CANCER

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ABSTRACT
A total number of 70 formalin-fixed paraffin embedded tissue blocks obtained from Iraqi patients with histopathologically diagnosed breast cancers, as well as 20 apparently healthy breast tissues (as a control group), were related to the period from January 2009 to August 2012, have been enrolled for CMV-DNA detection by in situ hybridization (ISH) technique. The mean age of patients was 41.7 years. CMV was demonstrated by ISH in (34.3%) breast cancer tissues (24 out of 70) which statistically show highly significant difference (p <0.001) when compared to the control breast tissues group (all were negative by ISH for CMV-DNA). Histopathological diagnosis has shown invasive ductal breast carcinomas in (54.3%) of cases , followed by the medullary breast carcinomas (28.6%) , then lobular breast carcinomas (17.1%) while the grading system revealed well grade breast carcinoma in 68.6% of cases followed by poorly grade (20%) and moderate grade (11.4%).

KEY WORDS: Human Cytomegalovirus, Breast Cancer, In Situ Hybridization.

INTRODUCTION

Breast cancer is relatively slow growing tumors and the most frequently diagnosed non –skin malignancy of women in many populations. The incidence increase with age, raising concern that it was an un identified environmental cause (Globocan 2008 & Iraqi Cancer Registry 2008). 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 (Iraqi Cancer Registry 2008). The three most studied viruses that could possibly cause breast cancer in humans are: mouse mammary tumor virus (MMTV), the Epstein-Barr virus (EBV) and the human papilloma (HPV) (Mant et al., 2004). MMTV and EBV occur in 37% and 50% of breast cancer cases, respectively (Lawson et al., 2001). One particular virus that has also been associated with breast cancer is human cytomegalovirus (HCMV). This virus is endemic in the human population and establishes lifetime latency in the host(Bluth,2010).HCMV binds to a broad range of cells, including most cells in which the virus does not replicate, engaging cellular surface heparin sulfate (Compton and Fiere, 2006; Mocarski, 2006). Human CMV infections have been implicated in etiology of several human malignancies, such as colon cancer, malignant glioma, adenocarcinoma of the prostate, EBV - negative Hodgkin's disease, carcinoma of oral cavity, Kaposi's sarcoma, Wilm's tumor, neuroblastoma, cervical carcinoma, and breast carcinoma (Kenny et al., 2002 & Rosenthal et al.,1993). Breast milk represents an established primary route of HCMV transmission in humans and the shedding of free virus occurs in the breast milk of 90% of women who are seropositive for HCMV (Hamprecht et al., 2001). 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 (Harkins et al., 2010). The genome of CMV is sufficiently large to encode over 200 proteins via 204 predicted open reading frames(ORF) . Of these , there was a direct evidence for a role of ORF 79 in transformation(Wolff et al., 1994)). HCMV- infected cells produce CMV-IL-10 protein, a viral homolog of the human interleukin-10 protein (hIL-10) (Bluth, 2010). It was shown that CMV-IL-10 activates Stat3 in human breast cancer cells through phosphorylation (pStat3). Stat3 has been identified as a key factor in tumor progression and evasion of programmed cell death (Behera et al., 2010). Many molecular methods are available for identification of nucleic acids of HCMV. Of these, in situ hybridization (ISH) can be used with frozen cells and tissues , cytological preparations and fixed tissues, using radioactive- labeled probes or probes with non-radioactive labels such as fluorescent moieties- biotin- digoxigenin- or enzyme- conjugated probes (Michaelism et al., 2009).

MATERIALS & METHODS

The study was designed as a retrospective one. It has recruited 90 selected formalin fixed, paraffin embedded breast tissue blocks, among them, (70) tissue biopsies from different grades of breast cancers and (20) apparently normal breast autopsies that were obtained from the archives of Forensic Medicine Institute / Baghdad and

used as control group. Following trimming process of these tissue blocks, the diagnosis of these tissue blocks were based on their accompanied records. Following trimming process of these tissue blocks, a consultant pathologist reexamined all these cases to further confirm their diagnosis.

One section was mounted on ordinary glass slide and stained with hematoxyline and eosin, while another slide was mounted on charged slide to be used for ISH for detection of CMV .The detection of CMV-DNA by ISH kit (US Biological. USA) was performed on 4μm paraffin embedded tissue sections using biotyinlated-labeled oligo-nucleotides probe which targets CMV-DNA.

For the in situ hybridization procedure, the slides were placed in 60 C° hot-air oven over night then the tissue sections were de-paraffinized and via then incubation of slides for 15 min (twice time) in xylene then treatment by graded alcohols via incubation for 5 min in 100% ethanol(twice time) .The same dewaxing protocols were routinely used for immunohistochemistry procedures, e.g. 15 min xylene (twice time), 5 min 100% ethanol(twice time), 5 min 96% ethanol(one time), 5 min 70% ethanol(one time), were used, finally immersion in distilled water for 5 minutes to remove residual alcohol. After that, slides were allowed to dry completely by incubating them at 37°C for 5 minutes. Then digestion process was done by adding proteinase K to the slides, then the slides were incubated at 37°C for 15 minutes. Then the slides were dehydrated by immersing them sequentially in the following solution at room temperature for the indicated times, distilled water for 1 minute, 70% ethanol for 1 minute, 95% ethanol for 1 minute and 100% by incubating them at 37% for 5 minutes. Then we add the 20 μl of cDNA probe added to each section and slides were covered by cover slips be careful to avoid trapping any air bubbles. After that probe and target DNA were denaturated by placing the cover slipped-slides in pre-warmed oven at 95°C for 8-10 minutes, slides were transferred to a pre-warmed humid hybridization chamber and incubated at 37°C for overnight. Then the slides were allowed not dry out at any time during the hybridization and staining. All reagents used during hybridization and detection were warmed to room temperature. At the next day, slides were soaked in pre-warmed protein block at 37°C until the cover slips fell off and should be careful not to tear the tissue, then the slides were allowed to remain in the buffer for 3 minutes, at 37°C after cover slips were removed. After that we add streptavidin-alkaline phosphatase conjugate reagent was added to tissue sections. Then slides were kept in a humid chamber at 37°C for 20 minutes. Then one to two drops of Slides were rinsed in detergent wash buffer for 5 minutes and then drained. After that One to two drops of 5-bromo3-chloro3-indoly/phosphate/nitro blue tertrazolium substrate-chromogensolution (BCIP/MBT) were placed on tissue section. Slides were incubated at 37 C° for 30 minutes or until color development was developed completed. Color development was monitored by viewing the slides under the microscope. A dark blue colored precipitate form at the complementary site of the probe in positive cells. Then the slides were rinsed in distilled water for 5 minutes, then counter staining process by immersion of the slides in Nuclear Fast Red stain for 30 seconds, then washing process was followed by immersion the slides for 1 minute in distilled water. After that Sections were dehydrated by ethyl alchol, (95%, once for one minute then, 100% twice times for 2 minutes each); cleared by Xylen, then mounted with permanent mounting medium (DPX).

The statistical analysis for significance of difference in the mean of normally distributed variables between more than 2 groups was assessed using ANOVA, while for parameters deviating from normal distribution; the nonparametric Kruskal-Wallis test was used.

RESULTS

The archival specimens collected in this study were related to breast cancer patients whom ages were ranged from sixteen years to seventy three years. The mean age of the patients with breast carcinoma (41.7 $\pm$ 10.4 years) was higher than the mean age of the healthy control (37.3 $\pm$ 9.7 years). Significant statistical difference ($p< 0.05$) was found between them according to age (Table1). The nuclear signals of HCMV- ISH were detected as blue discoloration at the site of complementary sequences. Table (2) & Figure (1) shows the positive results of HCMV DNA-ISH detection ,where 34.3% (24 of total 70) breast cancers showed positive signals. None in control group has presented positive signals for HCMV-ISH test. The most common histopathological type among all studied cases was ductal type (54.3%;38 cases) , followed by the medullary type (28.6%;20cases) , then lobular type (17.1%;12 cases) (Table 3). The statistical analysis of typing distribution of breast carcinoma shows significant difference ($p<0.05$) between ductal and lobular breast carcinomas, while non-significant difference was noticed between medullary and lobular breast carcinomas. The highest percentage (21.4%) of HCMV – ISH reactions was within ductal breast carcinoma, followed by medullary type of breast carcinoma (8.6%), then lobular type (4.3%).

TABLE 1: Distribution of breast cancer patients and healthy control group according to their age

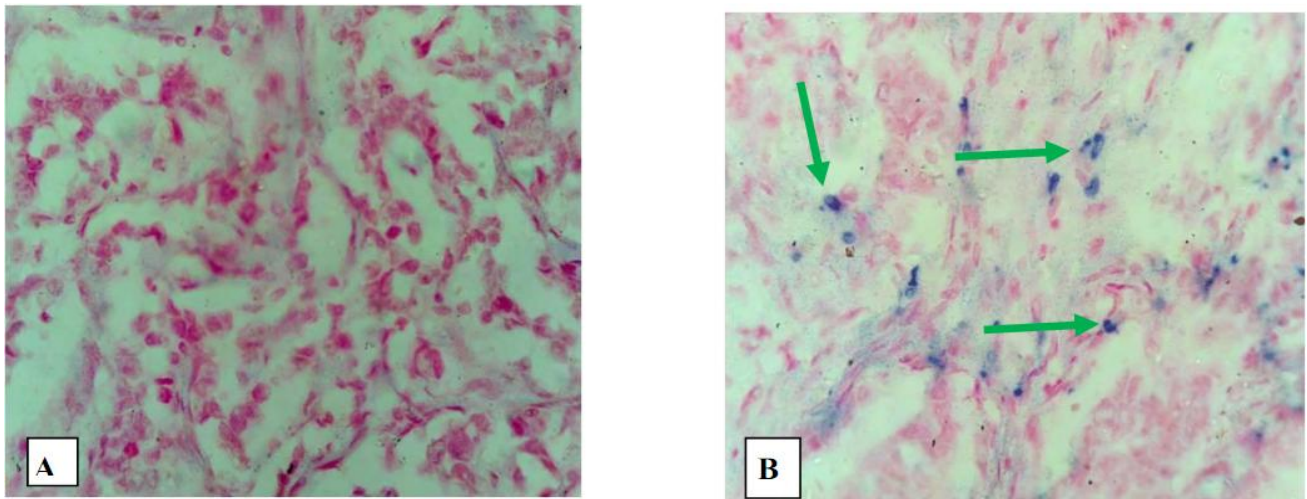| Study Groups	Number	Mean Age (Years)	Standard Deviation	Standard Error	Minimum (Years)	Maximum (Years)
Breast Cancers	129	41.7	10.4	1.09	16.0	73.0
Control	20	37.3	9.7	2.12	17.0	62.0
Statistical Analysis				(P = 0.007)		

FIGURE 1 :In Situ Hybridization (ISH) for HCMV Detection using biotinylated -labeled HCMV probe ;Stained with NBT/ BCIP (Blue)and counter stained by nuclear fast red(Red).A. Invasive breast cancer with negative HCMV –ISH reaction(40X). B. Invasive breast cancer with positive HCMV-ISH reaction that revealed low score and moderate signal intensity (40X).

TABLE 2 : Frequency distribution of HCMV- DNA ISH signal scoring among the breast cancers

HCMV-DNA signal scoring		Breast Cancers Group (n=70)		Normal Breast Tissues Group (n=20)		P
		N		N		
Negative		46/70	65.7	20	100.0	0.001*
Positive		24/70	34.3	0	0.00	
Scoring	I	8/24	33.3	0	0	
	II	10/24	41.7	0	0	
	III	6/24	25.0	0	0	

*Statistically the difference between breast cancer and healthy breast tissues groups for positive CMV cases was highly significant (P Kruskal-Wallis = 0.001).

In the present study, the highest percentage of CMV score signaling (41.7%: 10 out of 24 cases) was found in the moderate score (score II),whereas 33.3% (8 out of 24 cases) and 25.0% (6 out of 24 cases) were found within low (score I) and high (score I) scores ,respectively. Statistically, significant differences (p<0.05) were found on comparing the percentage of HCMV-DNA in the BC group according to their positive signal scoring.

TABLE 3: Frequency distribution of CMV DNA-ISH results according tohistopathologicaltyping of breast carcinoma.

Histopathological types of breast carcinoma		HCMV-DNA-ISH		Total
		Positive	Negative	
Ductal	Count	15	23	38
	% within Type	39.5%	60.5%	100.0%
	% within CMV	21.4%	32.9%	54.3%
Medullary	Count	6	14	20
	% within Type	57.1%	42.9%	100.0%
	% within CMV	8.6%	20%	28.6%
Lobular	Count	3	9	12
	% within Type	20.8%	79.2%	100.0%
	% within CMV	4.3%	12.8%	17.1%

Statistically, no significant correlations between HCMV infection and breast cancer typing .According to the Scarf-Bloom-Richardson system(SBR) for grading of breast cancers, the results of present study show that well differentiated grade breast carcinomas constituted 59.8% (48 of total 70 cases) , whereas cases with moderately and poorly differentiated grades constituted 20.2% (14out of 70 cases) and 11.7% (8 out of 70 cases) , respectively(Table 4) . In this study, the percentage of HCMV-DNA was found to decrease with the proceeding of the grading of breast cancer. Their HCMV-DNA-negative BC counterparts tissues were found to have a similar decreasing trend of grades of BC. The statistical analysis of grading distribution of HCMV-DNA in breast carcinoma shows significant difference (p<0.05)between well differentiated grade and poorly differentiated grade, while non-significant difference was noticed between

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poorly differentiated and moderately differentiated breast carcinomas .

TABLE 4: Frequency distribution of CMV DNA-ISH results according tohistopathologicalgrading of breast carcinoma

Grade / Differentiation		HCMV-DNA-ISH		Total
		Positive	Negative	
Poor	Count	6	2	8
	% within Grade	75%	25.0%	100.0%
	% within CMV	8.8%	2.9%	11.7%
Moderate	Count	8	6	14
	% within Grade	57.1%	42.9%	100.0%
	% within CMV	11.4%	8.8%	20.2%
Well	Count	10	38	48
	% within Grade	20.8%	79.2%	100.0%
	% within CMV	14.9%	54.9%	69.8%

DISCUSSION

Up to our best knowledge, this is the first work in Iraq with a molecular design that used a recent sensitive version of in situ hybridization technique to demonstrate the DNA of the late gene of HCMV in Iraqi patients with different grades breast cancers. In situ hybridization methods for detection of nucleic acid sequences have proved powerful especially for revealing genetic markers and gene expression in a morphological context (Kenny *et al*., 2002) .In addition and by courtesy of many previous studies that used ISH techniques had proved ISH as an effective method for detecting and localizing CMV-DNA within the affected tissues. Here about, it has been chosen the molecular design so as to demonstrate the HCMV-DNA of the late gene (that encodes matrix protein of this virus) in Iraqi patients with breast cancer with different grades. In the current study, the CMV DNA-ISH was detected in 34.3% (24 out of 70 cases) malignant breast tumors. None of control group presented positive signals for CMV-ISH test.

Unexpectedly, Harkins *et al*., 2010 was found 97% (31 out of 32 cases) of breast carcinoma in their study have evidence of HCMV infection where their expression was based on immunohistochemistry. From in vitro assays , three regions on the HCMV genome with transforming activity have been identified (Rosenthal *et al*., 1993). Multiple HCMV gene products are known to promote mutagenesis and to dysregulate cell cycle checkpoint controls and drive oncogenic signaling pathways (Michaelis *et al*., 2009). Breast feeding is the major route of HCMV transmission during the first year of life in countries where most women are seropositive and breast feeding their infants (Stagno and Cloud, 1994). Richardson *et al*., 2004 showed 60% of cases with breast cancer had antibodies to HCMV. HCMV could be associated with breast cancer because it is a ubiquitous virus that is shed in breast milk, as well as in saliva, urine, cervical secretion and semen, which implies that HCMV persistently infects epithelial cells (Sissons *et al*., 2002).

The low score categories HCMV this may be reflect low reproduction (replication) rate of the virus in breast epithelial cells. By analogy to this, it was found that high viral load of HPV was associated with a 3- to 5- fold increase risk for the development of cytologic high-grade squamous intraepithelial lesions(SIL of cervix& breast carcinomas during a follow –up period of up to 8 years compared with low viral load (Schlecht *et al*., 2003). However , if HCMV infection does contribute to tumor induction in humans, the mechanism underlying HCMV induced oncogenesis is very likely different from these DNA viruses that are known to play a role in human malignancies .

In Iraq, breast cancer is considered the most common type of malignancy among women accounting for about one third of the registered female cancers (Michaelism *et al*., 2009).This could be attributed to hereditary, environmental and life style factors. Most of Iraqi patients are diagnosed in younger age groups with late stage at presentation and a prevalence of more aggressive tumor behavioral forms (Stagno and Cloud, 1994)**.** Hormonal factors (namely, estrogen and its derivatives) are known to be involved in breast carcinogenesis where estrogen receptor – positive breast carcinomas are more sensitive to tamoxifen, an anti-estrogen (Pike *et al*., 1993). On reviewing the 70 cases which were included in this study, it was found the age of the patients with breast cancers was ranging between 15-75 years and their mean age was 41.7years .The present results are consistent with those reported world-wide where these breast malignant tumors were usually affecting females over forty years of age (Jean *et al*., 2005). These results could reflect that age is an important risk factor in tumor changes affecting breast epithelial tissues lesions. In general, aging increase the incidence of the malignant changes in breast epithelial tissues and as such their incidence was found to increase with age (Jean *et al.,* 2005*;* Elkum *et al.,* 2007).

In this study, a percent of 54.3%, 28.6%, 17.1% for ductal, medullary, lobular BC, respectively, were documented. A similar trend of highest frequency for

infiltrative ductal carcinoma was reported in an earlier studies in Iraq (Matloob,2006;Yalda, 2009). In addition, Albrektsen and co-workers (2010) also showed that ductal breast carcinoma represented the most common histopathological type in their study where their findings are also in agreement with the results of the present study. The HCMV-infected BC tissues in the present study were at highest percent in the ductal type of BC (21.4%) followed by medullary and lobular types (6.8% and 4.3%, respectively). The HCMV-non-infected BC tissues have showed the same trend of BC- type associated distribution. The results of Tsai *et al.*, 2005 (who found 76%; 47 out of 62 cases with invasive ductal breast carcinoma have evidenced HCMV expression) were incompatible with our results. There is no conclusion or frank evidence for BC type- associated effects of HCMV infection in the present results and our results are supporting the findings of Fisher *et al.* (1997) who found that patients who have invasive BC ductal type usually acquired more aggressive biological behavior and are more likely to developelympho- vascular invasion.

Despite of variability of grading systems (because of its subjective evaluation) yet, the most popular grading system used is Notingham modification of Scarf-Bloom-Richardson (SBR) system which depends on tubular formation, nuclear pleomorphism and mitotic figures (Leslie *et al.*, 2006).In the current study, it was found that breast carcinoma with well grades constituted (68.6%) followed by poorly grade (20%) and moderate grade (11.4%). These results are in disagreement with Zubair *et al.*, (2009) who showed that only 4.17% of cases have grade I, while grade II breast cancers were having (75.8%). However, grade III- breast cancers were constituted (20%) and are compatible with the present results.

Histological grading is an important parameter of risk assessment in the patients with breast cancer (Latinovic and Hinzc, 2001). In addition, other researchers have reported that tumors in younger women were of higher grade, with higher proliferation fractions, had more vascular invasion and expressed fewer estrogen and progesterone receptors compared to tumors in older women (Harvey *et al.,* 2004; Michael *et al.,* 2000). In this respect, it has been reported that the 10-year survival rate for patients harboring Grade I BC is around 80%, dropping to 45% in Grade III- breast cancers (Rosai and Ackermans, 2004). In this study, the percentage of HCMV-DNA was found to decrease with the proceeding of the grading of breast cancer. Their HCMV-DNA-negative BC counterparts tissues were found to have a similar decreasing trend of grades of BC. The present results support the conclusions of Yang *et al.*, 2004 that HCMV might not directly be involved in the oncogenic processes , but in addition to viral induction of tumor, it might enhance the possibility of oncogenesis.

CONCLUSIONS
The results suggest an important role for human cytomegalovirus in the development of breast cancer in our Iraqi patients, though its relation to the   histological grading denied to be regarded as a significant parameter of risk assessment in the patients with breast cancer.

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