Immunohistochemical Localization of Epstein Barr Virus-Latent Membrane Protein 1 in Breast Cancer Tissues

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ABSTRACT:

BACK GROUND:

Epstein- Barr virus (EBV) is well known to infect the vast majority of the world's adult population and has recently been reported in many literatures for its association with a broad spectrum of benign and malignant tumors, including breast cancer.

OBJECTIVE:

To analyze the relations and impact of Epstein Barr Virus- latent membrane protein 1(EBV-LMP 1) expression on a group of tissues with primary invasive breast cancers.

PATIENTS AND METHODS:

Forty- nine (49) formalin-fixed, paraffin- embedded breast tissues were obtained in this study; (34) biopsies from breast cancers (BC) and (15) from apparently normal breast autopsies control group. Detection of expression of Epstein Barr Virus- latent membrane protein 1(EBV-LMP 1) was done by HRP/DAB immunohistochemistry detection kit (an immunoenzymatic antigen detection system for immunohistochemistry techniques) using specific primary antibodies for EBV-LMP 1.

RESULTS:

Detection of EBV-LMP 1 - immunohistochemical (IHC) reactions in tissues with BC was observed in 11 out of 34 (32.4%), while in healthy breast tissues in the control group was detected in 13.3% (2 out of 15). The difference between the percentages of EBV-LMP 1 detection in BC tissues & control group was statistically not significant (P >0.05).

While no significant differences were observed between EBV-LMP 1 detection in BC tissues & the age of patients as well as grade of invasive breast cancers (P value > 0.05).

CONCLUSION:

Our results indicate that EBV could have a role in the pathogenesis of subset of breast cancers and contribution in their carcinogenesis.

KEY WORD: breast cancer, epstein barr virus, ebv - latent membrane protein 1, immunohistochemical technique.

INTRODUCTION:

Breast cancer is the most frequently diagnosed non –skin malignancy affecting women in many populations world-wide.[1,2,3]

Local studies in Iraq have demonstrated that most breast cancer patients presented in advanced stages with a likely prevalence of more aggressive tumor forms [4,5]. Many risk factors have been associated with breast cancer development and progression including a possible viral etiology [6,7].

Although various reproductive and hormonal factors have been identified as risk factors for breast cancer, yet these factors together do not explain more than fifty percent of all cases of breast cancer [8].

Researchers are thus prompted to consider other routes and risk factors, including a possible viral etiology, breast cancer pathogenesis.Three viruses that could possibly related to human breast cancers are: mouse mammary tumor virus (MMTV), the Epstein-Barr virus (EBV) and the human papilloma (HPV) [9].

A possible breast cancer association with EBV was raised for the following observations: (1)Reports of high male breast cancers incidence in EBV - endemic Mediterranean countries;[2] some EBV associated lymphomas are known to occur in breast; [3]morphological similarities between breast medullary carcinoma and EBV associated-nasopharyngeal carcinoma; (4)EBV has also been

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detected in breast tissue and milk; [5] lymphoblastoid cell lines bearing EBV can infect mammary epithelial cells in vitro; and [6]transfection of EBV DNA can lead to immortalization of epithelial cells, including mammary epithelial cells [6,9,10].

EBV and MMTV were reported in 50% and 37% of breast cancer cases ,respectively (Lawson et al., 2001) [11].

The current study is aiming to unravel the EBV-association with breast cancer in a group of Iraqi women.

MATERIALS AND METHODS:

Study Groups:

This study was designed as a retrospective research. Therefore, a collective number of (49) formalin-fixed, paraffin embedded breast tissue blocks enrolled in this study which comprised both patients and control samples whom their age ranged from 38 to76 years. These retrospective paraffin-embedded samples were retrieved from the archives of the period from 2011 till 2014 belonging to major hospitals and private histopathological laboratories in Baghdad. The diagnoses were based on their accompanied pathological reports of the corresponding patients. These blocks included a group of (34) biopsies from patients who had undergone surgical operation or biopsies for their breast cancers (BC) and (15) autopsies from apparently normal breast tissues control group. . These breast tissues were properly subjected to fixation as well as paraffin embedding and used for this research work as an age- and sex- matched groups.

Laboratory methods:

Tissue sectioning was conducted following trimming process of the tissue blocks at the histopathological department of Teaching laboratories / Medical City and a second confirmatory histopathological re – evaluation of each obtained tissue blocks was done by a consultant pathologist. One paraffin embedded (4 mm) thick-tissue section was prepared and mounted on ordinary glass slide and stained with hematoxyline and eosin, while another (4 mm) thick-tissue section was stuck onto positively charged slide to be used for detection of EBV-LMP 1– antigen using Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC kit (Lot. Number: ab64264) that was purchased from (Abcam, UK) , an immunoenzymatic antigen detection system for immunohistochemistry techniques, using specific Rabbit Monoclonal primary Anti-EBV Latent Membrane Protein 1 antibody [Lot. Number: D24-G; ab136633], that was also purchased from (Abcam, UK).The details of methods for performing IHC reaction with these antibodies were conducted according the instructions of that manufacturing company, and were done in the Research Laboratory of the Clinical Communicable Diseases Research Unit, at College of Medicine, University of Baghdad

Histopathological Analysis:

According to the specification of the kit, proper use of this ISH detection system gives an intense blue signal at specific sites of the hybridization probe in positive test tissues (by using light microscope).

The signal was evaluated under light microscopy using × 100 lens for counting the positive cells. The ISH results were given intensity and percentage scores based on intensity of positive signals and number of cells that gave these signals, respectively.

Positive cells were counted in 10 different fields of 100 cells for each sample and the average percentage of positive cells within the 10 fields was determined. A scale of 0-3 was used for relative intensity with 0 corresponding to no detectable ISH reaction, and 1, 2, 3 equivalents to low, moderate, and high intensity of reaction respectively. Cases were assigned to one of the following percentage score categories: 1%–25% (score 1), 26%–50% (score 2) or > 50% (score 3) (12).

STATISTICAL ANALYSIS:

T test, ANOVA test, and Chi square were applied for statistical examination of results obtained in our research. All these statistical analysis were done by using Pentium-4 computer through the SPSS program (version-10) and Excel application.

RESULTS:

The archival specimens collected in this study were related to breast cancer patients whom mean age was (52.8+ 8.6 years) while the mean age of those in the healthy control group was (61.8 + 6.9 years)(Table 1).

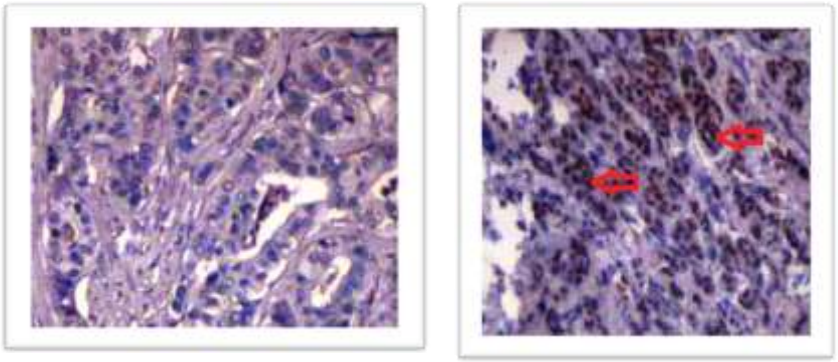
Table 1: Distribution of mean age (years) among the studied groups.

Type	Mean	95% Confidence Interval for Mean		Std. Deviation	Minimum	Maximum
		Lower Bound	Upper Bound			
Patients (N=34)	52.7941	49.8089	55.7793	8.55563	38.00	69.00
Control (N=15)	61.8000	57.9679	65.6321	6.91995	54.00	76.00

The signals of EBV-LMP 1 immunohistochemical reactions were detected as brown discoloration at the antigenic sites that were detected by their specific primary antibodies (Figure 1). Table (2) shows the positive results of EBV-LMP 1 - immunohistochemical (IHC) reactions, where 32.4% (11 of total 34) breast cancers showed positive signals while 2 out of 15 (13.3%) in control group has presented such positive signals for IHC test. The statistical Pearson Chi-Square analysis shows no significant differences among patient and control groups regarding EBV-LMP 1 immunohistochemical results (p =0 .117).

Table 2: Frequency of EBV-LMP 1 immunohistochemical reactions among the study groups.

			EBV		Total
			Positive	Negative	
Type	Patient	Count	11	23	34
		% within Type	32.4%	67.6%	100.0%
	Control	Count	2	13	15
		% within Type	13.3%	86.7%	100.0%
Total		Count	13	36	49
		% within Type	26.5%	73.5%	100.0%



A **B**

Figure 1: Immunohistochemical reactions (IHC) for detection of EBV-LMP 1 using specific primary antibodies for EBV-LMP 1 and biotinylated -labeled anti-EBV ;Stained with HRP/DAB (brown) and counter stained by hematoxyline (Blue).A. Invasive breast ductal carcinoma with negative EBV-LMP 1 –IHC reaction(40X). B. Invasive breast cancer with positive EBV-LMP 1 –IHC reaction that revealed moderate score and high signal intensity (40X).

In the present study, the highest percentage of EBV-LMP 1 score signaling (45.5%: 5 out of 11 cases) was found in each of the mild and moderate scores (score1 & 2), whereas 9.0% (1 out of 11 cases) was found with high scores(score 3) . Statistically, significant differences ($p<0.05$) were found on comparing the percentage of EBV-LMP 1 in the BC group according to their positive signal scoring (Table 3).

Table 3: Frequency distribution of signal scoring of EBV-LMP 1 immunohistochemical reactions among the breast cancers tissues.

`EBV-LMP 1 –IHC reaction		Frequency	Valid Percent	Cumulative Percent
Valid	Low score	5	45.5	45.5
	Moderate score	5	45.5	90.9
	High score	1	9.0	100.0
	Total	11	100.0	
Missing		23		
Total		34		

The highest percentage of EBV-LMP 1 signal Intensity in the present study was (45.5%: 5 out of 11 cases) that is related for each mild and moderate Intensity, while 9.0% (1 out of 11) of the breast cancers tissues were presented with strong intensity . Significant statistical differences ($p<0.05$) were found among breast cancers tissues according to their EBV-LMP 1- scoring intensities (Table 4).

Table 4: Distribution of signal- scoring intensities of EBV-LMP 1 immunohistochemical reactions in the examined breast cancer tissues.

EBV-LMP 1 –IHC reaction		Frequency	Valid Percent	Cumulative Percent
Valid	Mild Intensity	5	45.5	45.5
	Moderate Intensity	5	45.5	90.9
	Strong Intensity	1	9.1	100.0
	Total	11	100.0	
Missing		23		
Total		34		

There was no statistical significant difference between the frequencies of EBV-LMP 1 immunohistochemical reactions according to the age of the study groups (Table 5) .

Table 5:Frequency of EBV-LMP 1 immunohistochemical reactions according to the age of the study groups.

	EBV	N	Mean	Std. Deviation	Std. Error Mean
Age	Positive	11	50.6667	7.57188	2.18581
	Negative	23	53.9545	8.99988	1.91878

In the current study, it was found that the percentage of EBV-LMP 1 - IHC test reactions in the examined breast cancer tissues that have well grades constituted (42.86%) followed by moderate grade (50.0%) and poorly grade (0%). The statistical analysis of grading distribution EBV-LMP 1 - IHC reactions in breast carcinoma shows non-significant differences (Figure 2).

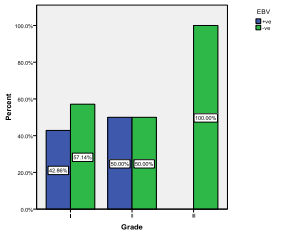


Figure 2:Distribution of EBV-LMP 1 immunohistochemical reactions according to the grading of examined breast cancer tissues.

DISCUSSION:

Breast cancers have ranked the top on the commonest ten cancers in the Iraqi provinces & districts, accounting for about one third of the registered female cancers [13] .

The majority of molecular events in the genesis of breast cancer are unknown [14]. Hereditary, hormonal (estrogenic hormones and derivatives), environmental and life style factors have been attributed to breast carcinogenesis [14].

High oncogenic-risk HPV genotypes (HPV 16, 18, 31 and 33)were detected in cases with breast cancer in a recent Iraqi study[15]. It has been reported that the association of high oncogenic-risk HPV types is stronger in invasive breast carcinomas, indicating a possible relationship with the pathology and grade of the disease [16].

The role of Epstein-Barr virus (EBV) in breast cancer etiology is still controversial. Unraveling this relationship is of potential importance in better understanding of breast carcinogenesis as well as early detection and / or prevention of such cancer.

In the current study, the results have shown EBV positivity in (32.4%) of BC tissues from Iraqi patients, (as represented by detection of EBV-LMP 1 - immunohistochemical reactions in BC tissues) while it was detected in 13.3% (2 out of 15) of breast tissues in the control group. EBV is among group-1 carcinogens as classified by IARC Working Group [17]. However, the viral prevalence vary markedly with the associated cancers, from about 10% in gastric carcinoma to nearly 100% in nasopharyngeal carcinoma [18–20], as well as the virus differs in the patterns of expressed viral genes, suggesting that EBV may affect cell growth in more than one way [20].

Thus, EBV represents an important but not a sufficient step in carcinogenesis, and an additional epidemiological risk factors could play a critical role in this process.

However, our results are comparable to the results reported by Zekri et al (2012)[21] where PCR for LMP1 was detected in 11/50(22%) of those 50 studied Iraqi cases and in 12/40(30%) of those 40 studied Egyptian cases who have invasive breast carcinoma.

Demographic features and genetic background might contribute to the global geographical differences. Nevertheless, other reasons for the different results reported in the literature may be attributable to the variation in the numbers of tested samples and sensitivity of the technical methods used in evaluation [22].

The origin of breast cancer remains multifactorial. But , if EBV is to be considered playing an etiological role in breast cancer development (as in the carcinogenesis of nasopharyngeal carcinoma and other strongly related cancers) and as an analogous to the expected role of HPV (as a group-1 carcinogen as classified by IARC Working Group, too) in the cervical cancers when compared to the role in HPV- associated breast cancers [9,15], it would be reasonable to expect that it would be possible to detect the virus at an early stage of the disease, and that EBV would be found at least in some normal tissues [9]. The fact that we found viral DNA in healthy breast specimens could

support, to a certain extent, the hypothesis that the virus might play a role in the etiology of breast cancer in only a subpopulation of patients. It is logical, on the other hand, to believe that the presence of EBV alone is not sufficient to implement the full carcinogenesis process and that further changes would accumulate over time in a stepwise manner to cause the disease and in turn suggesting a need for further large cohort studies to explore the role of each contributing factors.

On reviewing the 34 patients with breast cancers included in this study, it was found that their age were ranging between 38-69 years and their mean age was 52.8 + 8.6 years. These results are consistent with those Iraqi as well as world-wide reported results which have found that breast malignant tumors are usually affecting females aged over forty years [5,21,23,24]. These data have pointed that age is an important risk factor in tumorigenesis affecting breast epithelial tissues. In general, aging factor increases the possibility of malignant changes noticed in breast epithelial tissues and as such their incidence was found to increase with age. Also these results could be related to the effect of long exposure of these patients to the hormonal changes [23].

Most of Iraqi patients diagnosed with breast cancer are of younger age and presented with late stage as well as more aggressive cancers. [4]. In addition, other researchers have also reported that tumors in younger women were of higher grade and proliferation fractions as well as had more vascular invasion compared to tumors in their counterpart older women [25].

Our results have shown that EBV positivity in BC tissues are consistant to those results (28%) of Zekri et al. (2012)[21] regarding their studied Iraqi patients, but are less than the results (45%) regarding their Egyptian counterparts. These observations might be related to the different distributions of demographic and population characteristics, a fact that reflected as difference in the presence of EBV in breast cancer tissues from Egyptian and Iraqi patients , and as stated by [26].

Despite of variability of grading systems (which depend on subjectivity), yet Scarf-Bloom-Richardson (SBR) system is the most popular grading system used (evaluation is depending on tubular formation, nuclear pleomorphism and mitotic figures) [27]. Grading of breast cancers in this research was done according to Notingham modification of the (SBR) system. Histopathological grading is an important parameter in risk assessment of breast cancer patients [28]. 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 [27].

In this study, the results show that the percentage of EBV-LMP 1 - antigens in the examined breast cancer tissues was found to decrease with the proceeding of the grading of breast cancer. Their EBV-LMP 1 - negative BC counterpart tissues were found to have a similar decreasing trend of grades of BC. Regarding the association between EBV positivity and tumor grade, there was no statistical significant difference between the presence of EBV and tumor grades in the examined breast cancer patients (P=0.104). Our results are supporting the findings of Zekri et al. (2012) [21] who found no correlation between EBV positivity and tumor grades in patients with breast cancer in two different Arabic populations

Regarding the association between EBV positivity and tumor grade, there was not any statistical significant difference between EBV presence and tumor grade in both populations.

The present results support the conclusions of Yang et al., 2004 that EBV might have a role in induction of breast tumor , although not directly be involved in the oncogenic process, but it might enhance the possibility of oncogenesis.

CONCLUSION:

Our results might point for a role for the EBV to contribute in the early stage of development of subset of breast cancers in our Iraqi patients, though its relation to the histological grading not reach a statistical significance**.**

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