Original Article

Molecular detection of Epstein Barr Virus in Women with Breast cancer

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Summary:

Background: Epstein Barr Virus (EBV) infection has been implicated in pathogenesis of several types of carcinomas such as nasopharyngeal carcinoma, gastric cancer and bladder cancer and has recently been associated with breast cancer.

Objective: To evaluate the relations between Epstein Barr virus-encoded small RNA (EBER) and breast cancer.

Methods: Twenty two cases of breast cancer were retrieved from the Al-Kadhimiya Teaching Hospital in Baghdad. Clinical data were analyzed from the medical records and formalin fixed, paraffin embedded tumor tissue were examined by Chromogeneic in situ hybridization (ISH) technique for the detection of EBER.

Results: The expression of EBER in tissues patients with breast cancer in the present study was 50% (11out of 22), where strong correlation was found between expression of EBER and patients with breast cancer. While not found significant differences between ISH expressions of EBER with age, type of cancer, grade and lymph node metastasis.

Conclusion: Based on the results of the current study, Epstein Barr virus infection plays a major role in the pathogenesis of breast cancer.

Key word: Breast cancer, Epstein Barr virus, Epstein-Barr virus-encoded small RNA, in situ hybridization technique.

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Introduction:

Breast cancer is a type of cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk [1]. The size, stage, rate of growth, and other characteristics of the tumor determine the kinds of treatment. Treatment may include surgery, drugs (hormonal therapy and chemotherapy), radiation and/or immunotherapy [2].Many agents including radiation, chemicals and viruses, have been found to induce human cancer [3]. Viral factors are the most important class of the infectious agents associated with human cancers [4]. It was estimated that 17-20 % of world wide incidence of cancers attributable to a viral etiology [5]. Epstein Barr virus is one of the viruses that have some unclear and controversial points in its ability to trigger the development of certain tumors [6]. Like Burkett's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, gastric carcinoma and post-transplant lymphoprolifereative disease [7]. The small untranslated RNAs EBER-1 and -2 are accumulated at high levels during all forms of latency and regulate apoptosis through different mechanisms. EBER-1 interacts with the interferon-inducible protein kinase R (PKR), and inhibits its activation by double-stranded RNAs, protecting infected cells from IFN-induced apoptosis [8].Research into its etiology has focused primarily on reproductive and other factors affecting circulating sex hormones and on genetic susceptibility. So this study designs to investigate the correlation between expressions of EBER RNA and different parameters like: age, grade, type of tumor and presence or absence of lymph node metastasis in breast cancer.

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Materials and Methods:

Breast samples: Tumor and healthy breast tissue was collected from 32 Iraqi women. Twenty two cases were confirmed to by breast cancer and ten as biopsy negative. The patient's samples were collected during the period from (2010-2011) from pathology laboratories of Al-Kadhimiya teaching hospital in Baghdad. All patients were women, ranging in age from (27to70) years, were included in this study.

Tissue processing: Hematoxylin and eosin-stained slides were reviewed in all cases; unstained paraffin sections were used for Chromogeneic in situ hybridization analysis by using Digoxigenin-labeled oligonucleotides which target Epstein Barr Virus-encoded small RNA (EBER) and detection kit of EBER (ZytoVision GmbH. Fischkai 1D-27572 Bremerhaven. Germany).

In situ hybridization: The 4nm thick paraffin sections from 32 breast tissue (22 cases positive and 10 as negative) were deparaffinized by xylene and dehydrated by graded alcohol concentration (100%, 95% and 70%) and distal water then treated with Pepsin Solution for 20-30 min at 37°C in a humidity chamber according to manufacture instruction (ZytoVision GmbH. Fischkai 1D-27572 Bremerhaven. Germany). Immerse slides in distilled water and blot off the water. Then the slides

with digoxigenin-labeled probe with EBER. Denature the slides at 95°C for10 minutes on a hot plate. Transfer the slides to a humidity chamber and hybridization was the carried out for 2 hours at 37°C for RNA-targeting probes. It is essential that the tissue/cell samples do not dry out during the hybridization. The slides were soaked in Wash Buffer TBS for 5 min to remove the cover slip, and then treated with AP-Streptavidin. One to two drops of NBT/BCIP were placed on tissue section and incubate for 30 minutes at 37°C in a humidity chamber; the latter was monitored by viewing the slides under the microscope. Colored precipitate will form at the site of the probe in positive cells. Slides were then counterstained using eosin and sections were mounted with a DPX. Finally Evaluation of the sample material is carried out by light microscopy by a pathologist at power 400.

Data analysis: Fisher's exact test and t-test were used to obtain statistically significant differences between two groups with $p<0.05$ being considered statistically significant.

Results.

The mean age of the patients with breast cancer was 46.8 years when comparing with healthy control group was 59.70 years as shows in Table (1), there was significant differences ($P<0.05$) noticed between both groups.

In the present study it was observed that breast cancer percentage was increased with the increasing age.

Table (1): Distribution of Mean age (years) Among The Studied Groups

Studied groups	N	Mean years	Std. Deviation	Mini.	Maxi.	Student (t-test)	
						P-value	Sig.
Healthy Control group	10	59.7	8.9	48	70	0.005	Significant (P<0.01)
Breast cancer	22	46.8	12.1	27	70		
Total	32						

In situ hybridization results: The results of ISH which demonstrated that 11 out of 22 (50%) with breast cancer cases were positive for EBER. While EBER was not detected in healthy control group. However the statistical analysis of the distribution of positive results which demonstrated that significant differences as shown in (Table 2 and Figure 1).

Table (2): The Percentage of EBV-1 ISH-detection tests in the Studied Groups.

EBER ISH tests results	Breast cancer	Healthy Control group	Comparison of Significance	
			P-value	Sig.
Positive	11(50%)	0	0.006	Significant (P<0.01)
Negative	11 (50%)	10		
Total	22 (100 %)	10 (100 %)		

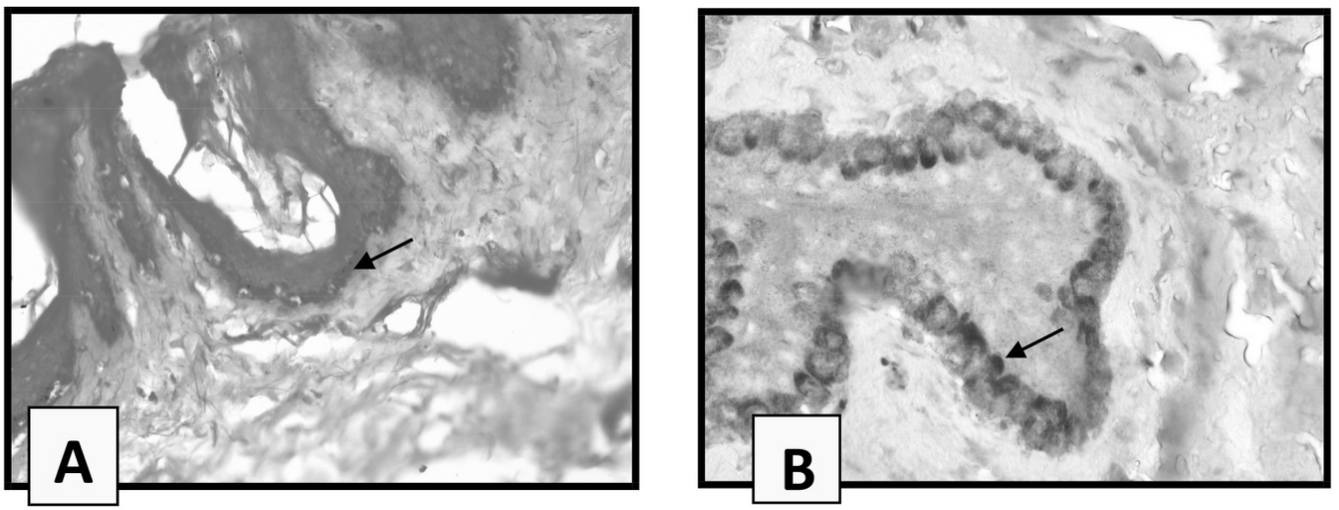

Figure (1): In situ hybridization staining of EBER in breast cancer section stained by DAB chromogen (brown) and counter stained with eosin (Magnification power, 400), A- EBER negative expression B- EBER positive expression.

Table (3) demonstrated the correlation between expressions of EBER with different variables. The results showed that there were no significant differences between ISH expression of EBER with age, type of cancer, grade and invasive of lymph node. Based on statistical analysis.

Table (3): In situ hybridization expression of positive and negative EBER RNA and related with clinicpathological profile of patients with breast cancer.

Variables		EBER positive	EBER negative	Comparison of Significance	
				Chi2-value	Sig.
Age	27-42	5 (45.5%)	3 (27.27%)	0.083	Non Sig. (P>0.05)
	43-58	5 (45.5%)	0		
	59-70	1 (9.1%)	8 (72.72%)		
Total number	22				
Types of tumour	Invasive ductal carcinoma	7 (63.6%)	5 (45.5%)	0.659	Non Sig. (P>0.05)
	Infiltrative ducal carcinoma	3 (27.3%)	5 (45.5%)		
	Recurrent carcinoma	1 (9.1%)	1 (9.1%)		
Total number	22				
Grade of tumour	I	3 (27.3%)	1 (9.1%)	0.135	Non Sig. (P>0.05)
	II	6 (54.5%)	10 (90.9%)		
	III	2 (18.2%)	0		
Total number	22				
Lymph node metastasis	Invasive	6 (54.5%)	7 (63.6%)	0.665	Non Sig. (P>0.05)
	Non invasive	5 (45.5%)	4 (36.4%)		
Total number	22				

Discussion:

Epstein-Barr virus-DNA and EBV-gene expressions have been shown in all malignant cells and therefore it is considered to have a pathogenic role [9]. In this study, investigated the association between EBER and breast cancer by in situ hybridization. The positive hybridization signals appeared mainly in the nuclei of infected cells. The probe used in the present study was designed to detect EBER, and results which demonstrated that EBER was observed in 50% of patients with breast cancer as shown in (Table 2 and Figure 1). This result was in agreement with the finding of ***Horiuchiet al.,*** [10]. Who determined the presence of EBV genome in patients with gastric and breast carcinoma. Labrecque et al., [11]. Which detected the EBV genome in 21% of DNA from 91 cases of breast carcinoma using polymerase chain reaction technique (PCR). Fina ***et al.,*** [12]. Who exploring a possible association between Epstein-Barr virus and breast cancer by large study consist of (509) breast cancers patients from different geographical areas by using polymerase chain reaction of a sub region of EBV BamHIC encoding the EBERs demonstrated that 31.8% of the tumours contained the viral genome also found no significant differences were observed among the geographical areas. Grintein ***et al.,*** [13]. Who investigated to the present EBV in different carcinoma of the breast, lung and other sites by using immunohistochemistry for detection of EBNA-1, in situ hybridization for EBERs and PCR Amplification and suggest that EBV is not restricted to lymphoepithelioma-like carcinomas but may play an oncogenic role in frequent epithelial cancers and possibly also in hyperplasias and certain dysplasias preceding carcinomas. Also our results was in agreements with other studies from different countries [14][15][16][17][18][19][20][21].The results of present study were disagreeing with Herrmann and Gerald [22]. Who investigated about EBV in (59) invasive breast carcinomas by using immunohistochemistry for the demonstration of the EBV-encoded nuclear antigen, in situ hybridisation for the detection of the EBV-encoded RNAs, and PCR for detection EBV DNA, the final result indicate that EBV-encoded RNA-specific in situ hybridisation and EBV-encoded nuclear antigen 1 immunohistochemistry were

negative in all cases. While during using the PCR, EBV DNA was detected in four out of 59 cases. Speck ***et al.,*** [23]. Who reported that no Epstein - Barr virus genomes in DNA from breast cancer-derived cell lines. DNA samples from 22 breast cancer-derived cell lines and from four non-breast cancer-derived cell lines were analyzed for the presence of EBV by polymerase chain reaction.

Regarding comparison of EBER expression results obtained according to age, the result revealed that the prevalence of EBER was found to be higher in age group 43-58(40%) than others as recorded in Table (3), this may be related to the risk of breast cancer is higher in middle-aged and elderly women than in young women [24].This risk increases as a woman ages, rising sharply after the age of 40. In the United States, more than three-fourths of all breast cancers occur in women aged 50 or older. Women who reach menarche at a relatively early age (12 or younger) and those who reach menopause at a relatively late age (55 or older) are slightly more likely than other women to develop breast cancer [25]. These relationships are believed to be mediated through estrogen production [26]. During the reproductive years, a woman's body produces high levels of estrogen. Women who start to menstruate at an early age and/or reach menopause at a late age are exposed to high levels of estrogen for more years than are women who have a late menarche or early menopause.

Age at first pregnancy is another aspect of reproductive history that is associated with breast cancer risk. Women who have their first full-term pregnancy at a relatively early age have a lower risk of breast cancer than those who never have children or those who have their first child relatively late in life [25].

The biologic basis for this relationship is not entirely clear. Women who were exposed to high doses of radiation, especially during adolescence, have an increased risk of breast cancer. This association has been observed both among atomic bomb survivors and among women who received high-dose radiation for medical purposes [27]. According to the age of patients with breast cancer most positive cases occur in the age groups (43-58).Regarding the histological grade the result revealed that the prevalence of EBER was found to be higher in grade II than other grade as recorded in Table (3). The results were in agreement with other studies which showed high frequency of moderately differentiated breast cancer [28] [29]. These results also could be explained due to limited sample size. According to type of cancer the present study demonstrated that most breast cancer occurred within invasive ductal carcinoma as shown in Table (3). This results agree with the findings of other researchers indicated the same results [12][28][29]. In conclusion, our results show the presence of EBER genome in a large subset of breast cancers. Because it is more frequently associated with the most aggressive tumours. EBV may play important a role in their development.

Reference :

1. Sariego, J: Breast cancer in the young patient. The American surgeon. 2010; 76 (12): 1397-1401.

1. *Florescu, A, Amir E, Bouganim N, Clemons M: Immune therapy for breast cancer in 2010-hype or hope. Current Oncology. 2011; 18 (1): 9-18.*

2. *De villiers, EM: Relationship between steroid hormone contraceptives and HPV, cervical interaepithelial neoplasia and cervical carcinoma. Int J Cancer. 2003; 103: 705.*

3. *Mao, C, Hughes JP and Kiviat N: Clinical findings among young women with genital human papillomavirus infection. Am J Obstet Gynecol. 2003; 188: 677.*

4. *Clifford GM; Smith S; Aguado T; Franceschi S: Comparison of HPV type distribution in high-grade cervical lesions and cervical cancer a meta-analysis. Br J Cancer. 2003; 89: 10.*

5. *Vokes E and Liebowitz DN: Nasopharyngeal carcinoma, Lancet. 1997; 350, Issue 9084: 1087-1091.*

6. *Thornhill Cher: Epstein Barr virus implicated in bladder cancer progression. Int J Urol. 2008; 15: 429-434.*

7. *Nanbo A, Inoue K, Adachi-Takasawa K and Takada K: Epstein-Barr virus RNA confers resistance to interferon alpha- induced apoptosis in Burkitt's lymphoma. Embo J. 2002; 21(5): 954- 965.*

8. *Abe T, Nobuo S, Mitsuhiro T, Toru H, Ataru S, Satoru Maruyama TM, Kenzo T and Katsuya N: Infiltration of Epstein-Barr virus harboring lymphocytes occurs in a large subset of bladder cancers. Int J Urol. 2008; 15: 429-434.*

9. *Horiuchi K, K Mishima, M Ohsawa, K Aozasa:Carcinoma of stomach and breast with*
lymphoid stroma: localisation of Epstein-Barr virus. Clin Pathol.1994; 47:538-540.

10. *Labrecque LG, Barnes DM, Fentiman IS and Griffin BE: Epstein - Barr virus in epithelial cell tumors: A breast cancer study. Cancer Res. 1995; 55: 39-45.*

11. *Fina F, Romain S, Ouafik L, Palmari J, Ben Ayed F, Benharkat S, Bonnier P, Spyratos F, Foekens JA, Rose C, Buisson M, Gérard H, Reymond MO, Seigneurin JM and Martin PM: Frequency and genome load of Epstein-Barr virus in 509 breast cancers from different geographical areas. Br J Cancer. 2001; 84(6):783-90.*

12. *Grinstein S, Maria VP, Paolo G, Paola AC, William HW, Elena DM and Victor EG: Demonstration of Epstein-Barr virus in carcinomas of various sites. American Association for Cancer Research. 2002; 62:4878.*

13. *Paul GM, David L, Jia J, Gillian D, Sukhjinder M, Andrew B, Judith T, David R, Christopher MC, Gary MR, Suk G, David E, Rebecca C, and Lawrence S Y: Reactivity with A monoclonal antibody to Epstein-Barr Virus (EBV) nuclear antigen 1 defines a subset of aggressive breast cancers in the absence of the EBV genome. Cancer Res. 2003; 63; 2338*

14. *Xue S A , I A Lampert, J S Haldane, J E Bridger and B E Griffin: Epstein-Barr virus gene expression in human breast*

cancer: protagonist or passenger. British Journal of Cancer. 2003; 89, 113-119.

15. *Glaser S L, Joe LH and Margaret LG: Epstein - Barr virus and breast cancer: state of the evidence for viral carcinogenesis. Cancer Epidemiol Biomarkers pre. 2004; 13:688.*

16. *Perkins RS, Katherine S, Cindy M, Diana DW, Gregory RP, Mark M, Nicholas J P, Irving MB, Patricia S, Virginie MA, Stephen PD and Leslie JK: Analysis of Epstein-Barr virus reservoirs in paired blood and breast cancer primary biopsy specimens by real time PCR. Breast Cancer Res. 2006, 8:70.*

17. *Preciado MV, Chabay PA, De Matteo EN, Gonzalez P, Grinstein S et al.: Epstein-Barr virus in breast carcinoma in Argentina. Arch Pathol Lab Med. 2005; 129: 377-381.*

18. *Fawzy S, Sallam M, Awad NM: Detection of Epstein-Barr virus in breast carcinoma in Egyptian women. Clin Biochem. 2008; 41: 486-492.*

19. *Trabelsi A, Rammeh S, Stita W, Mokni M, Mourou A et al,.: Detection of Epstein-Barr virus in breast cancers with lymphoid stroma. Ann Biol Clin. 2008; 66: 59-62.*

20. *Izham N S AS, H Zuridah, S Rashidah, O Azizon and N. A. Zeenathul: In situ hybridization and polymerase chain reaction methods for the detection of Epstein-Barr virus RNA in breast cancer specimens. African J Microb Res. 2011; 5(25): 4392-4397.*

21. *Herrmann K and Gerald N: Lack of evidence for an association of Epstein-Barr virus infection with breast carcinoma. Breast Cancer Res. 2003, 5:13-17.*

22. *Speck P, David FC and Richard L: Absence of the Epstein-Barr virus genome in breast cancer derived cell lines. J Natl Cancer Inst. 2003; 95 (16): 1253-1254.*

23. *Edwards BK, Howe HL, Ries LA, Thun MJ, Rosenberg HM, Yancik R, Wingo PA, Jemal A, Feigal EG: Annual report to the nation on the status of cancer, 1973-1999, featuring implications of age and aging on U.S. cancer burden. Cancer 2002, 94 (10): 2766-2792.*

24. *Helmrich SP, Shapiro S, Rosenberg L, Kaufman DW, Slone D, Bain C, Miettinen OS, Stolley PD, Rosenshein NB, Knapp RC, Leavitt T Jr, Schottenfeld D, Engle RL Jr, Levy M. Risk factors for breast cancer. Am J Epidemiol. 1983, 117 (1): 35-45.*

25. *Henderson BE, Ross RK and Bernstein L: Estrogens as a cause of human cancer: The Richard and Hinda R: Foundation Award Lecture. Cancer Res. 1988, 48: 246-253.*

26. *Preston DL, Mattsson A, Holmberg E, Shore R, Hildreth NG, Boice JD: Radiation effects on breast cancer risk: a pooled analysis of eight cohorts. Radiat Res. 2002, 158 (2): 220-235.*

27. *Mathilde B, Jean-Marc G, Elisabeth K, Virginie G, Ellen B, Genevieve C, Irene J: Detection of Epstein-Barr Virus in invasive breast cancers. J Nati Cancer Institute. 1999; 91(16):1376-1381.*

28. *Deepti J, Munira Q, Neha G, Rajnish J, Nitin G: Association of Epstein-Barr virus infection (EBV) with breast cancer in rural indian women. 2009; 4(12): e8180.*