Journal of the Egyptian National Cancer Institute 29 (2017) 77–82

Contents lists available at ScienceDirect

Journal of the Egyptian National Cancer Institute

journal homepage: www.sciencedirect.com

Full length article

Epstein-Barr virus infection and breast invasive ductal carcinoma in Egyptian women: A single center experience

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ARTICLE INFO

Article history:
Received 14 October 2016
Received in revised form 25 February 2017
Accepted 25 February 2017
Available online 24 April 2017

Keywords:
Epstein-Barr virus
Breast invasive
Ductal carcinoma
Nested PCR
Immunohistochemistry

ABSTRACT

Background: A controversy of the role of Epstein-Barr virus (EBV) infection in breast carcinomas has been reported in the literature.
Objectives: We carried on this research to explore possible association between EBV infection and breast invasive ductal carcinoma (IDC) in Egyptian women attending our center.
Study design: This study carried out at Sohag university hospital on 84 paraffin embedded samples of breast tissue, of them 42 breast IDC as the case group and 42 breast fibroadenomas as the control group. Nested PCR and immunohistochemistry (IHC) done separately for all samples to identify the Epstein-Barr nuclear antigen-1 (EBNA-1) gene and EBV latent membrane protein-1 (LMP-1) respectively, in breast cancer cells and controls.
Results: Specimen considered positive when both (EBNA-1) gene and LMP-1 were detected using PCR and IHC separately for the same sample, this was achieved by 10/42 (23.81%) of breast IDC (case group) and 6/42 (14.29%) of breast fibro-adenomas (control group) (P-value = 0.4). Nodal involvement was the only parameter that demonstrated a significant statistical relationship with EBV presence in cancerous tissue with p-value = 0.003.
Conclusion: Our research could not find a significant statistical association between EBV infection and breast IDC in Egyptian women attending our center, but, there might be an association between the existence of EBV and tumor aggressiveness.

Introduction

Breast cancer is the most common malignancy of females in many populations and the second leading cause of death in the world [1]. According to National Cancer Institute (NCI) Egypt registry data, female breast cancer ranked first among tumors and IDC the most frequent pathological subtype.[2]

The identification of a viral agent for breast cancer has eluded researchers for decades [3,4]. As for many decades, oncogenic viruses have been hypothesized as having potential causal roles in breast cancer. The main candidate viruses include EBV, high risk human papilloma virus (HPV), mouse mammary tumor virus (MMTV) – like envelope DNA and Cytomegalovirus (CMV) [5–7]. Co-infection with more than one of these viruses has been argued; as the probability of a single patient to be infected with two or more distinct types of viruses is increasing [8,9].

Co-infection of EBV and HPV seems to be present in a significantly higher proportion in breast cancer than in normal breast epithelial cells. The Glenn group reported that HPV and EBV coexist in several human cancers; and the presence of these viruses in breast cancer is associated with young age at diagnosis and, possibly, an increased breast cancer grade [10,11].

The first report on the role of EBV in breast cancer has been described by Labrecque et al. 1995 [12]. Then accumulated reports and observations have strengthened this role; for example the high incidence of breast cancer in Mediterranean countries with endemic EBV infection, EBV -associated lymphomas in the breast and the morphological similarities between breast medullary

Peer review under responsibility of The National Cancer Institute, Cairo University.
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http://dx.doi.org/10.1016/j.jnci.2017.02.002
1110-0362/

carcinoma and EBV-associated nasopharyngeal carcinoma [3]. Nevertheless; data on viral presence and oncogenic mechanisms are still inconsistent and detailed mechanisms of interactions between infectious agents and host cells have yet to be fully elucidated [13]. But it has been suggested that cell cycle proteins could be the target of EBV- transformation mechanism, like other oncogenic viruses [14]. EBV uses its viral proteins; the actions of which mimic several growth factors, transcription factors, and anti-apoptotic factors, to usurp control of the cellular pathways that regulate diverse homeostatic cellular functions [15]. For example; the Epstein–Barr virus (EBV) nuclear antigen (EBNA)-1 promotes the accumulation of chromosomal aberrations in malignant B cells by inducing oxidative stress and activation of the DNA damage response [16,17]. In addition, EBV-positive neoplasms show genetic alterations that are distinct from those exhibited by EBV-negative neoplasms; for example EBV-positive gastric adenocarcinoma that displays recurrent PIK3CA mutations, extreme DNA hypermethylation, and amplification of JAK2, CD274 and PDCD1LG2 [18]. A recent work on DNA cytosine deaminases suggested that APOBEC3B -a newly defined source of DNA damage- has a role in breast cancer development. It showed that viral infections causing innate immune responses and/or splice variants may be contributing factors for its action [19]. In support of this idea; recent studies on head/neck cancer have linked human papilloma virus infection to APOBEC3B upregulation and implicated APOBEC3B mutagenesis in activation of PIK3CA kinase which is mutated in a large proportion of breast cancers [20].

Molecular techniques are the most definitive assays in establishing viral presence in cancerous tissue in comparison to other tests based on host antibody assessment. Up to date, no standard method has been generally accepted for EBV detection in cancer tissues, PCR and IHC have been considered as the most sensitive methods [21].

We conducted this study to explore possible relationship between EBV infection and breast IDC using both IHC and PCR techniques.

Materials and methods

Samples and data collection

Specimens obtained from pathology department laboratory at Sohag university hospital. Biopsies obtained by incisional and/or excisional method. Specimens were formalin fixed, and paraffin embedded. Collectively we have obtained84 paraffin embedded blocks; 42 IDC not otherwise specified (NOS) (as case group) and 42 fibroadenomas (as control group). Clinical data obtained from medical records of those patients. These data include: age, histopathological grade, tumor size, hormonal receptor status and TNM categories besides to other factors.

The study has been carried out under Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Informed consent taken from participants and the study approved by ethical committee of Sohag faculty of medicine.

Detection of EBV by nested PCR

DNA extraction

Using a microtome; 8 sections of 5–10 μm thickness cut up from blocks and immediately placed into 2 ml microcentrifuge tube. Paraffin dissolved and DNA extraction done using QIAamp® DNA FFPE Tissue (Qiagen, USA) according to the manufacturer instructions.

Testing DNA integrity by standard PCR

The integrity of the extracted DNA confirmed by standard PCR using β-globin primers G073 (5′GAAGAGCCAAGGACAGGTAC-3′) and G074 (5′CAACTTCATCCACGTTCACC-3′). Reaction volume of 25 μl (supplied by Invitrogen; Groningen, the Netherlands, UK) used containing 2.5 μL of 1X PCR reaction buffer, 1 μL DNA solution, 1 μL MgCl2, 2 μL of each of the gene-specific primer, 0. 125 μL Taq-DNA polymerase, 2.5 μL deoxynucleoside triphosphates mix (dNTPs), and 16 μL PCR water. PCR amplifications performed on a T-Gradient thermal cycler (Biometra, USA). PCR amplification conditions as follows: initial heating at 95 °C for 10 min; followed by (denaturation at 95 °C for 5 min, annealing at 58 °C for 30 s; extension at 72 °C for 30 s) for 35 cycles then final extension at 72 °C for 5 min. Samples with negative β-globin gene excluded from the study.

Nested PCR

Two primer sets described by Cinque et al. were used. The first round for amplifying a 297 bp fragment (EB3 5′-AAG GAGGGTGGTTTGGAAAG), (EB4 5′-AGACAATGGACTCCCTTAGC); while the second one using a primer set that binds within the first round product generating a 209 bp fragment (EB1 5′-ATCGTGGTCAAGGAGGTTCC, EB2 5′-ACTCAATGGTGTAAGACGAC). The first cycle conducted using a reaction volume of 25 μl (supplied from Invitrogen; Groningen, the Netherlands, UK) containing 2.5 μL of 1X PCR reaction buffer, 1 μL DNA solution, 1 μL MgCl2, 2 μL of the first set gene-specific primer, 0. 125 μL TaqDNA polymerase, 2.5 μL deoxynucleoside triphosphates mix (dNTPs), and 16 μL PCR water. The cycling conditions as follows: initial heating at 95 °C for 3 min., followed by 35 cycles of 95 °C for 30 s, followed by 55 °C for30 s, then 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR product of the first cycle used as the template for the second cycle where 1 μl of it added to complete the 25 μl volume mixture as mentioned before; then placed in the thermal cycler under the same cycling condition of cycle 1. PCR amplicons separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide with a marker (DNA Ladder 100 bp, 10 bands) from (KOMA BIOTECH, Seoul, Korea) to assess PCR product size; then the bands photographed by a gel documentation system (Ingenius, Syngene, USA) (Figs. 1 and 2).

Positive control is genomic DNA which isolated from EBV-positive Hodgkin's lymphoma case. Negative control is PCR with the omission of the DNA template (Figs. 1 and 2).

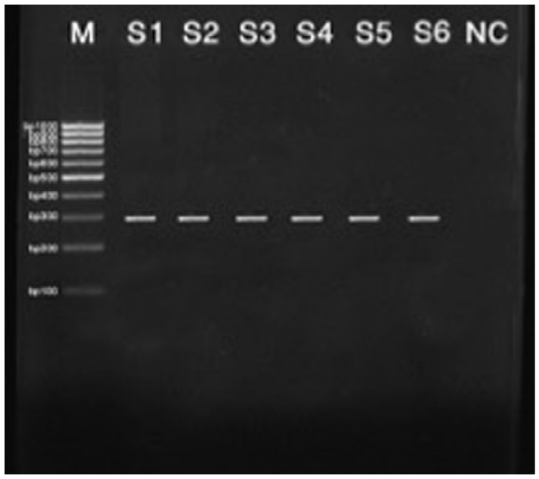

Fig. 1. agarose gel electrophoresis showing the amplified PCR product of β globin gene (297 bp) in S1–S5 indicating intact DNA of the corresponding specimens. NC: negative control; M: marker.

Fig. 2. agarose gel electrophoresis of the nested PCR product showing the bands EBNA-1gene (209 bp) in S1–S5. M: marker; NC: negative control; PC: positive control.

Detection of EBV by immunohistochemistry

All samples subjected for IHC staining, pre-cleaned (Superfrost®) */Plus-Fisherbrand ®-USA) slides used. Formalin-fixed paraffin-embedded sections immune stained using peroxidase-labelled streptavidin-biotin technique to detect Epstein-Barr virus expression. Mouse monoclonal antibody to Epstein-Barr virus/ LMP Ab-1Cat# MS- 1458-S0 (0.1 ml) LABVISION Corporation used. All specimens stained with the antibody (Figs. 3 and 4).

Staining procedure

Anti-polyvalent HRP-DAB detection system Cat# TPD-015 (15 ml) was used. Tissue sections de-paraffinized in 2 changes of xylene and re-hydrated through descending grades of alcohol and washed in distilled water. Endogenous peroxidase activity blocked with hydrogen peroxide (Cat# DHP-xxx) using peroxidase blocking reagent and then washed in 20% diluted phosphate buffered saline (PBS). Slides immersed in antigen retrieval solution (10 mmol sodium citrate buffer solution, pH 6.0) and ovened at 100°c for 90 min; finally, they were washed in distilled water and in PBS. Tissue sections incubated in 1/50 Epstein-Barr virus in 1/100 normal goat serum (NGS) overnight at room temperature to block nonspecific interactions. After rinsing in PBS, tissue sections treated with biotinylated anti-goat serum for 10 min at room temperature. The slides rinsed and peroxidase-labelled streptavidin applied for 10 min at room temperature, rinsed again with PBS and blotted. The slides incubated with 14-diaminobenzidine

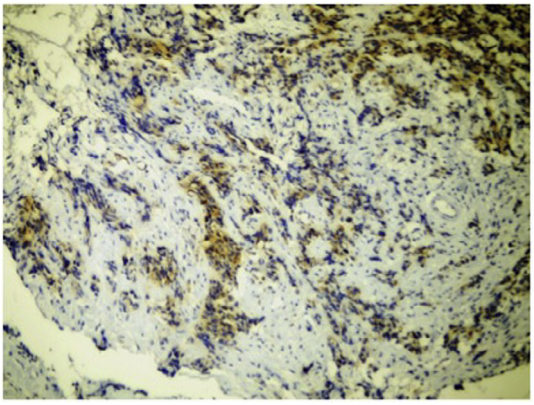

Fig. 3. Infiltrating ductal carcinoma not otherwise grade III specified shows moderate cytoplasmic staining for EBV/Ab-1 (X200).

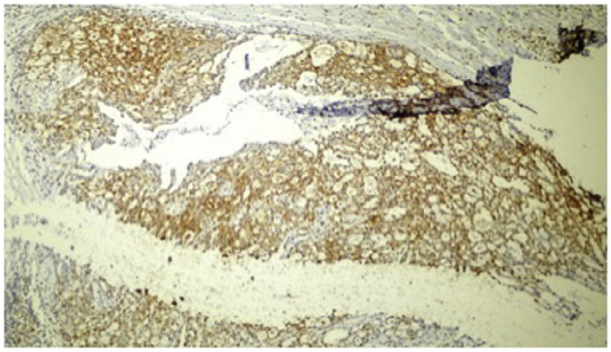

Fig. 4. Infiltrating Ductal carcinoma not otherwise specified grade II shows moderate cytoplasmic staining for EBV/Ab-1 (X200).

(DAB) and 0.06% H2O2 for 20 min, washed in distilled water and counter-stained using Myer's Hematoxylin. Tissue sections washed in tap water dehydrated in ascending grades of alcohol, cleared in xylol, left to dry, then mounted with DPX, and cover slipped.

Positive controls: Sections from EBV-positive Hodgkin's lymphoma. Negative controls: more sections of the examined tissues stained in parallel, but with omission of the primary antibody.

Positive cases identification

All samples were tested by PCR and IHC separately and independently; only positive cases by both samples were identified as positive EBV, we would like to emphasize that the PCR results were identical for IHC results except for 2 cases where they were positive by PCR and negative by IHC and these were not considered as positive one and excluded.

Statistical methods

Data was analyzed using STATA intercooled version 12.1. As all data were qualitative data, they were presented as number and percentage and compared using Chi square test and Fisher's exact test. P value was considered significant if it was less than 0.05 and was highlighted for each test (Table 2).

Results

Clinical and pathological features of the study group

The clinical characteristics of our study include: age, histopathological grade, tumor size, hormonal receptor status and TNM categories. These parameters analyzed with EBV detection; age of IDC cases ranged from 23 to 70 years with a median age of 43 years; while fibroadenomas control group age ranged from 24 to 50 years and median age 34 years. 61.9% of the patients in IDC case group were less than 50 years of age with a median of 43 years; while in fibroadenomas group it was 34 years. The most frequent grade was GII (61.9%) while 52.38% of these tumors were of 3–5 cm range in size. Nodal involvement by cancer detected in 66.7% of IDC cases with one third of them having N1 stage. Estrogen receptors negative in 57.14% of these cases and only 2 cases were metastatic.

EBV detection results

By nested PCR technique; EBNA-1 gene detected in 12 out of 42 (28.57%) IDC specimens, while detected only in 6 out of 42 of fibroadenoma specimens. By combining IHC technique for all study

Table 1
Comparison between the studied groups as regards detection of EBV by PCR and IHC.

Group	PCR		IHC	
	+ve	–ve	+ve	–ve
Cases	12/42 28.57%	30/42 71.42%	10/42% 23.81%	32/42 76.19%
Controls	6/42 14.29%	36/42 85.71%	6/42 14.29%	36/42 85.71

Table 2
Pathologic and clinical features of patients with BC and their relation to EBV detection.

Characteristics	No of patients	(%)	Detection of EBV by IHC		P value
			Yes	No	
Age					
<50	26	(61.90%)	4 (15.38%)	22 (84.62%)	0.10*
≥50	16	(38.00%)	6 (37.50%)	10 (62.50%)	
Grading					
GI/GII	28		4 (14.29%)	24 (85.71%)	0.06*
GIII	14		6 (42.86%)	8 (57.14%)	
Tumor size					
<3	4	(9.52%)	2 (50.00%)	2 (50.00%)	0.06
3–5	22	(52.38%)	2 (9.09%)	20 (90.91%)	
>5	16	(38.10%)	6 (37.50%)	10 (62.50%)	
LN					
Negative	14	(33.33%)	2 (14.29%)	12 (85.71%)	0.31
Positive	28	(66.67%)	8 (28.57%)	20 (71.43%)	0.45*
T					
T1/T2	26		4 (15.38%)	22 (84.62%)	
T3/T4	16		6 (37.50%)	10 (62.50%)	0.14*
N					
N0/N1	28		2 (7.14%)	26 (92.86%)	0.003
N2/N3	14		4 (28.57%)	10 (71.43%)	0.15*
M					
M0	40	(95.24%)	10 (25.00%)	30 (75.00%)	1.00*
M1	2	(4.76%)	0	2 (100%)	
ER					
Negative	12	(28.57%)	4 (33.33%)	8 (66.67%)	0.46
Positive	24	(57.14%)	4 (16.67%)	20 (83.33%)	0.51*
Unknown	6	(14.29%)	2 (33.33%)	4 (66.67%)	
PR					
Negative	10	(23.81%)	2 (20.00%)	8 (80.00%)	0.82
Positive	26	(61.90%)	6 (23.08%)	20 (76.92%)	0.77*
Unknown	6	(14.29%)	2 (33.33%)	4 (66.67)	

* Means analysis carried out by Fisher's exact test; no * means by Chi square test.

samples including those with PCR positive results; LMP-1 protein of EBV expressed on 10/42 IDC specimens – which were 10/12 of PCR positive samples; – representing 23.81% and expressed on 6/42 of control samples – which were 6/6 of PCR positive fibroadenoma specimens. There was no a significant statistical difference between the detection of EBV in breast IDC and fibroadenoma samples (P value = 0.4) as presented in Table 1.

EBV positivity and clinico-pathological features relationship

As regard the association between EBV positivity and characteristics of IDC group; only nodal involvement showed statistically significant association as possibility to detect EBV increases with more involved nodes (p-value = 0.003) (Table 2).

Discussion

Breast cancer prevalence has been reported differently in the world. EBV as a putative factor for breast cancer- risk or casual- has been debated over the past decades; first link in this issue has been proposed by Labrecque LG, et al. in 1995 when they found about 50% of tested breast cancer materials positive for EBV DNA [11]. Then Bonnet et al. in 1999 using PCR detected EBER-2 and LMP-2 DNA in 51% of breast cancer cases [22]. It was very difficult to distinguish EBV disease from background infection [23]. In many studies, a joint approach has been applied to overcome the limitation of methods used for EBV detection in breast cancer [22].

Yet the results stay unconvincing, and their interpretation has been a matter of debate for years. This variation is due to the failure of some studies to find EBV in breast carcinoma [22,24]. A possible explanation might be the epidemiological variation in EBV infections; such as variance in age at the time of acquiring primary EBV infection and socioeconomic background; as populations with higher incidence rates of breast carcinoma correspond to those with higher possibility of delayed primary EBV infection [25]. Furthermore, this controversy might be due to diversity in the methodologies used for detecting the virus and different EBV-derived proteins or nucleic acids investigated. In addition; loss of the virus episome during cell division of malignant cells may result in absence of viral DNA 'hit and run' behavior [26].

Our study was not designed to compare the two techniques; but to define the most probably positive case of EBV infection inside the tumor cell or in breast cells in control. Positive cases by either technique alone excluded. We tried to detect EBV in breast tissue specimens by combining 2 methods; nested PCR using 2 primer sets for EBNA-1 gene and IHC using antibodies against EBV/ LMP-1 protein used separately to test all specimens; then we identified EBV case as the sample that is positive in both tests. EBV detected in 12/42 and 10/42 0f IDC samples by PCR and IHC, respectively. None of negative PCR results was positive by IHC, so, we considered only positive cases by the 2 methods. For control group; both methods showed the same detection of 6/42 samples.

As regard the tested specimens; we used formalin-fixed paraffin-embedded specimens as many studies carried out for the same purpose [24,27]. But, one study with both frozen and formalin-fixed paraffin-embedded specimens was negative to detect EBV by PCR but 5% of specimens were positive by nested PCR [28]. One study of fresh breast cancer tissue samples was a positive study [29].

Genetic basis studies of phyllodes tumor identified DNA copy number alterations including both gains and losses, but with significant heterogeneity seen among these cases [30–32]. Studies of fibroadenoma have shown much lower frequency of chromosomal alterations than in phyllodes tumor [31]. Another study showed that fibroadenomas have the highest and lowest expression of epithelial- and proliferation-related genes, respectively, whereas malignant phyllodes showed the opposite expression pattern. Within the dataset of IDCs and normal breast tissues, the vast majority of fibroadenomas identified as Normal-like by intrinsic breast cancer subtyping. For that, fibroadenomas can be considered as intermediate phenotype between normal and malignant breast lesions; so we used fibroadenomas as control group [33].

In our work; nested PCR showed EBV DNA detection in 28.57% IDC specimens (case group) and 14.29% of fibroadenoma specimens (control group); this is in agreement with Arbach H, et al. who were able to detect EBV DNA in 46% of breast tumors by PCR [34,35]. Also, a study from UK showed EBV DNA in 34% of breast cancer tissue [36]. But, in other studies detection was less than ours as in an Iranian study where it was 7.3% [37] and in Herrmann et al. – Germany; it was (6.8%) [24], while in USA; Perrigoue JG and Deshpande CG were able to detect EBV DNA in 45% [38] and 42% [39] of specimens, respectively. A recent Sudanese study showed EBV DNA in 55.5% of breast cancer cases [40].

By IHC; we found EBV virus LMP-1 protein in 23.81% of IDC specimens and in 14.29% of fibroadenoma specimens (P value 0.4%); this finding approaches findings of two Egyptian studies; Fawzy et al. and Mohamed et al. [27,41]. They reported EBV proteins in 25% and 35.3% of invasive breast carcinoma specimens, respectively. Our findings were in agreement with a Jordanian study that showed EBNA-1 protein in 26% of breast carcinoma cases [42]; but not in agreement with an Iranian study which showed LMP-1 protein in 7.5% of the studied cases [43]. But; these studies including ours had certain limitations as they have looked for expression of only one viral protein. They should have investigated multiple EBV proteins present in different phases of viral latency seen in other EBV associated tumors.

Marked variability in PCR and IHC findings were noticed in many studies done over the last two decades [12,11]; moreover, EBV infection has been found most consistently and in a prevalence ranging between 7.5 and 55%. All these studies could not produce evidence of a pathogenic role of EBV in breast carcinomas.

Apparent variability of these findings should not itself exclude a possible role for EBV in breast carcinogenesis; as this is influenced to some extent by two issues: first, the marked variation in EBV prevalence even among studies using similar techniques [22,44]; second, the possibility of false-positive or false-negative analytical test results due to lymphocyte-derived EBV, cross-reactivity immune-stains, amplicons contamination, or inappropriate technique sensitivity [45].

In our study; only lymph node involvement showed significant association with the presence of EBV in cancerous tissue. These findings come in agreement with two Egyptian studies [27,41] which suggest aggressive tumor behaviour. Also; this matches findings by Bonnet et al. [22] who showed that EBV detection in primary tumors varied by nodal status (P = 0.01), largely because of the difference between subjects with more than three lymph nodes versus those with less than or equal to three lymph nodes involvement (72% versus 44%). Again this matches results found by Arbach et al. [35] who showed that EBV infection of breast tumor enhances its mutagenic properties, such as invasion, angiogenesis, and metastasis.

In contrary to our study; a recent study carried in UK debates this finding as it concluded that EBV positivity was not associated with grade, hormonal receptor status, or disease stage [36].

Conclusion

Our study could not prove a statistically significant association between EBV infection and occurrence of breast IDC tumors; but a possible association between EBV detection and tumor aggressiveness might be present.

Recommendations

A past priority has been to confirm the identification of EBV virus in breast tumors. The new priority is to determine whether EBV is a causal factor or not; rather than innocuous passenger invading pre-existing malignant tissues. So, executing another research with larger number of samples with pan marker in all stages of EBV infection is mandatory.

Conflicts of interest

None.

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