Molecular Detection of Epstein-Barr virus in Breast Cancer
Howida M. Sharaf¹ and Mostafa F. Gomaa
Clinical Pathology Department ¹, Ain Shams University Hospitals
and Gynecology & Obstetrics Department, Faculty of Medicine, Ain Shams University,

Abstract

Background: The association of Epstein Barr virus (EBV) with breast carcinomas (BCs) is still in controversy. The aim of this study was to clarify the association of EBV & BC in Egyptian females and to assess its role as potential contributor to the development and behavioral alteration of BC.

Subjects & methods: EBV-DNA was detected using PCR on breast tissue from 40 female patients with primary invasive BC; ductal (n=32) and lobular (n=8) and 20 age matched females undergoing reduction mammoplasties as control.

Results: EBV-DNA was detected in 8/40 (20%) BC specimens. On the other hand all control specimens were negative. As regards prognostic factors, no association was observed between EBV-DNA and patients' age, menopausal status and steroid receptor expression. However, significant associations were detected between the presence of EBV-DNA and other poor prognostic factors. All of the EBV-DNA positive BC were significantly associated with positive nodal status, where 7/8 cases showed more than three tumor-positive LN involvement.

In spite of the small number of invasive lobular carcinoma included in this study there was a significant correlation between this histological type of poor prognosis and EBV-DNA detection rate where 4/8 (50%) of them were positive for EBV-DNA compared to 4/32 (12.5%) detection rate in invasive ductal carcinoma. A significant correlation was found between EBV-DNA detection rate in BC and high tumor grade of invasive ductal carcinoma; (100%, 1/1) association with grade III versus (9.67%; 3/ 31) with grade I.

Conclusions: Our results demonstrated the presence of the EBV genome in a considerable subset of BC in Egyptian patients. The virus was more frequently associated with bad prognostic factors. This indicates that EBV may play a role in the development and behavioral alteration of some aggressive BC.

Keywords: Epstein Barr Virus, breast cancer.

Introduction

As the etiology and progression of breast cancer remain incompletely understood, novel routes of disease pathogenesis are important to consider. Viral pathogens have not been much explored, but recent interest has focused on Epstein-Barr virus (EBV). EBV is a γ herpes virus; its 184-kb DNA genome encodes approximately 100 genes that infects >90% of humans and is usually carried lifelong as an asymptomatic infection. It is the causative agent of infectious mononucleosis (IM) and has been associated with a growing list of malignancies of both lymphoid and epithelial origin including Burkitt's lymphoma, B-cell lymphoma in immunocompromised, Hodgkin's lymphoma, and
nasopharyngeal carcinoma (NPC). Based on this association, the WHO International Agency for Research on Cancer (IARC) has classified EBV among group I carcinogens which are agents that definitely cause neoplasm in humans [Sally et al., 2004 & Ribeiro-Silva et al., 2004].

Over the past decade several investigators have raised the possibility that EBV may also be involved in the pathogenesis of breast carcinoma (BC), the most common carcinoma in females [Murray, 2006]. Early studies addressing this issue focused on medullary carcinomas since these are morphologically similar to NPC. However, these studies consistently failed to detect EBV using various techniques [Lespagnard et al., 1995 & Dadmanesh et al. 2001]. The possibility that invasive ductal and invasive lobular BC might be EBV-associated was raised by Labrecque et al. [2001], triggering a large number of follow up studies. However, this association has been constantly debated and the results of different studies are controversial [3]. Proof beyond a reasonable doubt that EBV had anything to do with the development of BC requires substantial additional evidence that can only be obtained through further research. In addition, various characteristics make the association of EBV with BC deserves to be further studied. First, IM affects mainly the teenagers, a period of strong proliferation of the mammary glands in girls [Godshall & Kirchner, 2000]. Second, in vitro, mammary epithelial cells can be infected by direct contact with lymphoblastoid cellular lines infected by EBV [Zur Hausen, 2009]. Third, the transfection of subfragment of EBV-DNA stimulates the growth of normal human breast epithelial cells [Gao et al., 2002]. Fourth, BC has epidemiologic similarities with young adulthood Hodgkin's disease (YAHD) that has an established causal association with EBV [Yasui et al., 2001]. Fifth, EBV has been identified in benign tumors of the breast among immunosuppressed females [Kleer et al., 2002]. Sixth, in vitro, EBV-infected cells are resistant to paclitaxel (taxol), a chemotherapy commonly used in the treatment of BC, and the virus provokes overexpression of a multidrug resistance gene (MDR1) [Arbach et al., 2006].

Finally, immunotherapeutic and antiviral strategies are currently being developed for the treatment of EBV-positive Hodgkin's lymphoma and NPC [Francisco et al., 2011 & Kast, 2006], and they could potentially also be applied to EBV-associated BC.

Based on these considerations and in view of the high incidence of female BC in Egypt [WHO report, 1997] this association required clarification as it could profoundly shape the clinical diagnosis, disease management and, potentially, patient outcome.

Accordingly this study was carried out to clarify the association of EBV & BC in Egyptian females and to assess its role as potential contributor to the development and behavioral alteration of BC.

**Subjects and methods**

This study included 60 Egyptian females divided into 2 groups:

- Breast carcinoma group:
  
  This included 40 female patients that had primary unilateral invasive BC with no other primary cancer and they underwent modified radical mastectomy with no preoperative neoadjuvant treatment at Ain Shams University Hospitals during 2008. Their age
ranged from 30–69 years with mean±SD 52.45±10.83. The majority were postmenopausal 28 cases (70%), while premenopausal cases were 12 (30%).

- Control group:
  This group included 20 age matched females undergoing reduction mammoplasties. All were subjected to the following:
  - Full clinical history, clinical examination of the breast, radiology (Mammography, a chest radiograph was done to detect chest metastasis and to assess chest condition before anesthesia)
  - Fine needle biopsy to diagnose cancer breast for patients.
  - Other routine investigations as blood picture, liver functions; renal functions and ECG were also done to detect any general disease that may modulate the line of treatment.
  - Intraoperative frozen section to confirm the preoperative diagnosis for patients.

**Histopathological examination for patients:**
Conventional histopathological examination. Tumor size was defined as the largest diameter of the tumor at the time of trimming of the fresh specimens. Surgical specimens were fixed in buffered formalin for 24 hrs to prepare paraffin blocks and hematoxylin and eosin stained sections for conventional histopathological examination (histological type, grade, lymph nodes status, and in situ component of the tumor).

Most prevalent invasive breast tumors were involved in this study; invasive ductal carcinoma (32 patients) and invasive lobular carcinoma (8 patients). Histological grading of invasive ductal carcinoma was determined according to the criteria of Scarff–Bloom and Richardson (SBR) [Le Doussal et al., 1989] regarding nuclear grade, extent of tubular formation, and highest mitotic count in a representative area of high-power field. No specific grading system is available for invasive lobular carcinoma [Bane et al., 2003]. All dissected lymph nodes (LNs) in the axillary specimen were examined for metastases to detect involved LNs/total dissected number and infiltration of the perinodal fat. Immunostaining for estrogen and progesterone receptors (ER and PR) were done.

**PCR amplification of EBV DNA:** Total DNA was extracted from approximately 50 mg of tumor & breast tissue using the DNeasy tissue kit (Qiagen Inc., California, USA), following the manufacturer’s protocol. Spectrophotometric quantitation of DNA was carried out at 260 nm.

The polymerase chain reaction (PCR) was used to amplify a 110 base pair DNA fragment in Bam HI-W highly conserved large internal repeat region within the EBV [Kassim et al., 1998], using primers, forward 5′-GTTCGCGTTGCTAGGCCACC-3′(1041–1060 bp) & reversed 5′-AGGACCACTTTATACCAGG-3′(1131–1150 bp)

PCR amplification was done in 50 μL total reaction volume containing 300ng of DNA or 50 ng of control, 100 pmol of each primer, 0.2mM of each dNTPs, 0.5 unit thermostable Taq DNA polymerase and 1X Reaction buffer (all reagents were supplied by Promega, USA). DNA positive control for EBV was derived from NPC known to harbor the virus and nuclease free distilled water replacing DNA was used as negative control.

Cycling conditions were performed as follows: one initial denaturation step at 94 ºC for 2 min; 30
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cycles of denaturation at 92º C for 2 min, annealing at 45 ºC for 1 min, and extension at 72ºC for 2 min; and one final extension step at 72 ºC for 10 min. Amplified DNA fragments were resolved by electrophoresis of PCR products in 2% agarose containing ethidium bromide (0.5 mg/ml) and visualized under UV transilluminator. The expected PCR products size was 110 bp (Fig 1)

**Statistical analysis**

All statistical analyses were performed using Statistical Package for Social Science (SPSS) version 12. Chi-square test was used to compare qualitative variables. Fisher's exact test was used to test the association of categorical variables. P≤0.05 was considered a significant difference.

**Results**

Pathologic and clinical features of patients with BC and their relation to EBV-DNA were illustrated in table 1. As regards patients with BC, thirty two patients (80%) had invasive ductal carcinoma, while invasive lobular was diagnosed in 8 cases (20%). According to SBR grading for invasive ductal carcinoma 31/40 cases (77.5%) were grade II and only one case was grade III (2.5%). As regards LN status, out of the 40 cases, 22 showed positive nodal metastases (55%) and 21/22 had N3 L.N involvement, while 18 cases showed negative nodal metastases (45%). As regards hormonal receptor study; ER showed mild immunoreaction in 24 cases (60%), 12 cases (30%) showed moderate nuclear reaction, and only two cases (5%) exhibited marked strong diffuse immunoreaction. Negative reaction was evident in two cases (5%). As regards PR; 26 cases (65%) showed mild immunoreaction, 8 cases (20%) and 2 cases (5%) showed moderate and marked nuclear immunoreactivity respectively.

EBV DNA was detected in 8/40 (20%) BC specimens. On the other hand all control specimens were negative. As regard prognostic factors, no association was observed between EBV-DNA and patients' age, menopausal status and steroid receptor expression. However, significant associations were detected between the presence of EBV-DNA and other poor prognostic factors. All of the EBV-DNA positive BC were significantly associated with positive nodal status, where 7/8 cases showed more than three nodal status, where 7/8 cases showed more than three nodal status.

In spite of the small number of invasive lobular carcinoma included in this study there was a significant correlation between this histological type of poor prognosis and EBV-DNA detection rate where 4/8 (50%) of them were positive for EBV-DNA compared to 4/32 (12.5%) detection rate in invasive ductal carcinoma. A significant correlation was found between EBVDNA detection rate in BC and high tumor grade of invasive ductal carcinoma; (100%, 1/1) association with grade III versus (9.67%; 3/ 31) with grade II.

EBV-DNA was not detected in all BC cases with tumor size <2 cm (T1). On the other hand all positive cases for EBV-DNA were of tumor size >2 cm (T2 and T3), however the difference was statistically insignificant.

In the control group, a significant statistical difference was detected between patients with BC and the control group as no EBV-DNA was detected in any specimen of the control group indicating that EBV is significantly restricted to BC (Table 2).
Table 1: Relationships between clincopathologic characteristics of patients with BC and EBV-DNA.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of Patients (n = 40)</th>
<th>(% )</th>
<th>Detection of EBV by PCR No. of +ve/ Total no. (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Menopausal state</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Premenopausal (30 – 49 years)</td>
<td>12</td>
<td>(30 %)</td>
<td>2/12 (16.6 %)</td>
<td>0.73 (NS)</td>
</tr>
<tr>
<td>Postmenopausal (50 – 69 years)</td>
<td>28</td>
<td>(70 %)</td>
<td>6/28 (21.4 %)</td>
<td></td>
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<tr>
<td><strong>Tumor size; cm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt; 2cm (T1)</td>
<td>10</td>
<td>(25 %)</td>
<td>0/10 (0 %)</td>
<td>0.12 (NS)</td>
</tr>
<tr>
<td>&gt; 2 cm (T2 &amp; T3)</td>
<td>30</td>
<td>(75 %)</td>
<td>8/30 (26.6 %)</td>
<td></td>
</tr>
<tr>
<td><strong>Histological type</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Invasive ductal</td>
<td>32</td>
<td>(80 %)</td>
<td>4/32 (12.5 %)</td>
<td>0.02 (S)</td>
</tr>
<tr>
<td>Invasive lobular</td>
<td>8</td>
<td>(20 %)</td>
<td>4/8 (50 %)</td>
<td></td>
</tr>
<tr>
<td><strong>Histological (SBR) grade</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G II</td>
<td>31</td>
<td>(77.5 %)</td>
<td>3/31 (9.67 %)</td>
<td>0.007 (S)</td>
</tr>
<tr>
<td>G III</td>
<td>1</td>
<td>(2.5 %)</td>
<td>1/1 (100 %)</td>
<td></td>
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<tr>
<td><strong>Steroid hormone receptor status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ER –ve</td>
<td>2</td>
<td>(5 %)</td>
<td>0/2 (0 %)</td>
<td>0.47 (NS)</td>
</tr>
<tr>
<td>+ve</td>
<td>38</td>
<td>(95 %)</td>
<td>8/38 (21 %)</td>
<td></td>
</tr>
<tr>
<td>PR -ve</td>
<td>4</td>
<td>(10 %)</td>
<td>2/4 (50 %)</td>
<td>0.12 (NS)</td>
</tr>
<tr>
<td>+ve</td>
<td>36</td>
<td>(90 %)</td>
<td>6/36 (16.6 %)</td>
<td></td>
</tr>
<tr>
<td><strong>Axillary LN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-ve</td>
<td>18</td>
<td>(45 %)</td>
<td>0/18 (0 %)</td>
<td>0.004 (S)</td>
</tr>
<tr>
<td>+ve</td>
<td>22</td>
<td>(55 %)</td>
<td>8/22 (36.3 %)</td>
<td></td>
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</tbody>
</table>

S = Significant, NS = None Significant

a: All tumors were carcinoma.
b: Scarff-Bloom & Richardson classification.
ER= estrogen receptor PR= progesterone receptor

Table 2: Prevalence of EBV in studied groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>PCR</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>+ ve</td>
</tr>
<tr>
<td>Patients</td>
<td>8/40</td>
</tr>
<tr>
<td></td>
<td>20 %</td>
</tr>
<tr>
<td>controls</td>
<td>0/20</td>
</tr>
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<td></td>
<td>0 %</td>
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P = 0.03
Discussion

Cancer remains a major public health challenge despite progress in detection and therapy. Breast cancer is the most common malignancy among females. Over the past decade, the EBV association with BC has been constantly debated despite the well-documented presence of EBV genetic material in up to 51% of breast tumors. This controversy is due to the failure of some investigators to identify EBV in BC [Murray, 2006]. This might be due in part to epidemiological variation in EBV infections, like difference in the age at which the studied patients had acquired primary EBV infection; as populations with higher incidence rates of BC correspond to those with higher likelihood of delayed primary EBV infection [Yasui et al., 2001]. In addition, this controversy might be due to differences in the methodologies used for detecting the virus and different EBV-derived proteins or nucleic acid analyzed. In the present study by using PCR targeting sequences specific for the Bam HI-W region of EBV, EBV-DNA has been detected in 20% of BC samples. Indeed, EBV has been identified most consistently using PCR as in studies done by Luqmani & Shousha [1995], Labrecque et al. [2001], Fina et al. [2001], Preciado et al. [2005], Tsai et al. [2005], and Perkins et al. [2006] with respective frequencies 20%, 40%, 51%, 31.8%, 35% and 45.2%. Among several studies using PCR for detecting EBV.
in BC, prevalence is noticed to be highest when PCR targeted Bam HIW or EBERs (Epstein–Barr Encoded RNA) sequences, more moderate when PCR targeted LMP1 or EBNA-4 gene, and lowest in examination of EBNA-1 gene; indicating the importance of the PCR target on the extent of the association [Glaser et al., 2004].

Furthermore, EBV-DNA was not detected in the specimens of the control group. These results confirm that the EBV was restricted to tumor cells. The major difference between cases and controls is strongly suggestive of a role for EBV in BC. This is supported by several studies that have used breast tissue either from normal women or from various benign diseases or from normal breast tissues adjacent to the tumor as controls; such latter tissues are more likely to carry suspect viruses than normal tissue sourced from normal women. EBV genetic material and / or gene products were rarely identified in control breast tissues and were restricted to tumor epithelial cells [Bonnet et al., 1999, Labrecque et al., 2001, Grinstein et al., 2002, Preciado et al., 2005 & Tsai et al., 2005]. Even when Chu et al. [2001] have found that there are more infiltrating lymphocytes in EBV-positive BC than in EBV-negative tumors (71% against 27%), these infiltrating lymphocytes themselves were EBV negative.

In a recent study using laser capture microdissection combined with real-time quantitative PCR, Arbach et al. [2006] have detected EBV genomes in approximately 50% of breast cancer specimens. They also found that the viral load was highly variable from tumor to tumor. Moreover, EBV genomes were heterogeneously distributed in morphologically identical tumor cells, with some clusters of isolated tumor cells containing relatively high genome numbers while other tumor cells isolated from the same specimen were negative for EBV-DNA.

From these results we can suggest that EBV may play a role in breast cancer oncogenesis but it is unlikely to be a primary etiological agent as EBV is only detected in some breast cancer cells. Instead, EBV mostly acts in concert with other co-factors. It may alter the behavior of already transformed cells so that they acquire a more aggressive phenotype. This hypothesis is supported by the observation that EBV-associated breast cancers are more commonly aggressive than other breast cancers [Murray et al., 2003] and by the current study where a significant correlation has been detected between invasive lobular carcinoma, the histological type of poor prognosis, and EBV-DNA detection rate. In addition, EBV genome was detected in tumors with size >2 cm (T2 and T3) and in high histological SBR grade of invasive ductal tumors (II and III), however, this needs further investigation as no significant correlation was found between the tumor size and the presence of EBV-DNA and all the studied invasive ductal BC were of high grade. The association between the presence of EBV genetic material and higher BC grade has been observed by Murray et al. [2003]. They also observed that EBV is detected more frequently in breast tumors that are hormone-receptor negative; pointing to the aggressiveness of these tumors. In contrast, no association was observed in this study between EBV-DNA detection and steroid receptor expression as the majority of the BC specimens studied expressed both ER and PR. The concept that EBV and related cancers are
negatively correlated with hormones may not be true. In studies conducted during the 1960s on African patients with apparent EBV-associated NC, it was noted that these patients had high urinary estrogen and testosterone excretion levels [Lawson et al., 2006]. These observations are compatible with the recent findings from studies in cattle, which show the presence of proteins that activate EBV transcription factors in exocrine and endocrine cells, including such cells in the lactating cow mammary gland [Broadhurst et al., 2005].

Adding to the poor prognostic factors, all tumors with EBV genome were significantly associated with positive nodal status, where 7/8 (87.5%) of them were associated with more than three LN involvement. This is in accord with Bonnet et al. [1999] who recognized similar finding. This association with axillary LN invasion suggests that the infection by EBV may be related to the high metastatic potentiality of these tumors. In 2001, Subramanian et al. discovered that EBV protein (EBNA-3C) can bind to and alter the function of a human metastatic suppressor protein called Nm23-H1, which normally suppresses the movement of malignant cells and is found in all human cells. When this natural brake on cell migration is disabled by the virus, cancerous breast and lymphatic cells are free to metastasize, or spread. If substantiated, this finding would have major implications regarding prevention and therapy of the disease. People with aggressive forms of cancer are most vulnerable and should be checked to determine the status of previous viral exposure when physicians are choosing the most appropriate treatment for them. It also would be wise to closely monitor people with a history of active EBV infection for early signs of cancer [Subramanian et al., 2002].

In conclusion, our results demonstrated the presence of the EBV genome in a considerable subset of BC in Egyptian patients. The virus was more frequently associated with bad prognostic factors. This indicates that EBV may play a role in the development and behavioral alteration of some aggressive BC. In the light of the new approaches in treating EBV-associated malignancies these results give a hope that a substantial percent of invasive BC could be treated with antiviral agents or with immunotherapy.

References


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المقدمة:
مكانت علاقة فيروس ابشتاين بار (EBV) وسرطان الثدي في النساء المصريات، وتقديم دوره المحتمل كمساند في حدوث وتغير سلوك سرطان الثدي

الحالات وطرق البحث:
تم الكشف عن دي إيبفيروس ابشتاين بار (EBV) باستخدام تقنية تفاعل البلمرة المتسلسل في نسبة الثدي من 40 من المرضى النساء المصابات بسرطان الثدي المبكر المختصر (32 من فصيلة الأخفية و8 من فصيلة المفصص) وذلك بالإضافة إلى 20 من الإناث المتطلبة العمرو اللائي يخضعن لعمليات تجميل بالثدي كمجموعة ضابطة.

النتائج:
ولقد أظهرت النتائج عن وجود دي إيبفيروس ابشتاين بار (EBV) في 20% من حالات سرطان الثدي. ومن ناحية أخرى كانت جميع العينات في المجموعة الضابطة سلبية. وبحسب مراقبة العوامل المترتبة، لم يلاحظ أي ارتباط بين المرض والدواء، وحالة انقطاع الطمث والتعبير عن الوراثة الشتاء للعنصر المشع يمكن وجبة تشويه في الوراثة المشعة للعنصر المشع، حيث أن نسبة من ثمانية حالات أظهرت وجود أكثر من ثلاثة عقد إيجابية. وعلي الرغم منقلة عدد السرطان المفصص المختصر المدرجة في هذه الدراسة، ومع ذلك، تم الكشف عن ارتباط نمو دالة إيجابية بين وجود دي إيبفيروس ابشتاين بار (EBV) وعمر المرض، ولكل اكتشاف دى إيبفيروس ابشتاين بار (EBV) كان 50% بالمقارنة إلى 12.5% في معدل اكتشاف سرطان الثدي المخالب. وقد وجد ارتباط كبير بين معدل اكتشاف دي إيبفيروس ابشتاين بار (EBV) في سرطان الثدي، وال Açور من الدرجة العامة من سرطان الثدي المخالب 100% من الصفر

الاستنتاج:
ورقد خلص هذا البحث إلى أن الجينوم لفيروس ابشتاين بار (EBV) ووجود في مجموعة فرعية لا أساس لها من مرضى سرطان الثدي في مصر. وكان الفيروس مرتبط بالعوامل المترتبة بسوء المال مشيرا إلى أنه قد يلعب دورا في حدوث سرطان الثدي وتغيير السلوكي لمبادلة سرطانات الثدي المشرسة.