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Localization of Epstein-Barr virus to infiltrating lymphocytes in breast carcinomas and not malignant cells

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ABSTRACT

The pathogenesis of breast cancer is unknown. In recent years, a number of studies have implicated a role for Epstein-Barr virus (EBV) in a subset of cases. However, these findings are controversial and others have failed to find any link between the virus and this malignancy. We hypothesized that technical differences and the different type and ethnic origin of the cases may be the cause of the disparities reported. Using a highly sensitive EBER-in situ hybridization and immunohistochemistry, we examined 219 samples (158 malignant and 61 non-malignant) from 61 Emirati breast cancer cases to determine if EBV was etiologically associated with Emirati cases and if there was any correlation with other established prognostic factors such as age, histological type, lymph node metastasis, estrogen, progesterone and HER2 expression. We found 47.5% of the cases to be EBV positive, but the virus was localized to occasional infiltrating lymphocytes and not in the malignant cells. EBV lymphocytes were more commonly observed in lymph nodes than in breast tissues, but there was no correlation with malignancy or hormone status. The mean age of our patients was 48 years and hormone receptor staining revealed 20% of the cases to be triple negative (ER−/PR−/HER2−). We conclude that although EBV can be detected in breast cancer cases, it is not directly associated with the disease. Thus, a PCR-based approach cannot be used to link this ubiquitous virus to the pathogenesis of breast cancer. Furthermore, we do not find any correlation between the presence of EBV in infiltrating lymphocytes and ER, PR, HER2 expression. We believe our findings will help explain some of the controversies relating to the role of EBV in the pathogenesis of breast cancer.

Introduction

Breast cancer is among the most common malignancy affecting Emirati women, and indeed women worldwide. The etiology of breast cancer remains unknown. A number of risk factors have been identified to be associated with the pathogenesis of this malignancy, including geographical location (more common in developed countries), early age of menarche, late age of both menopause and late first full-term pregnancy, family history of breast cancer and life style (Veronesi et al., 2005). However, in as much as 50% of the cases, no identifiable risk factor can be identified. This has prompted investigators to examine the possibility that an oncogenic virus may be involved (Amarante and Watanabe, 2009).

Arguably, one of the best studied human oncogenic viruses is Epstein-Barr virus (EBV). EBV is a lymphotropic herpesvirus, implicated in the pathogenesis of a number of human malignancies of both epithelial and lymphoid origin, including Burkitt's lymphoma (BL), post transplant lymphoproliferative disorders (PTLD), undifferentiated nasopharyngeal carcinoma (NPC) and Hodgkin lymphoma (HL) (Rickinson and Kieff, 2006). More recently, a number of studies have suggested that EBV may also play a role in the pathogenesis of breast cancer (Magrath and Bhatia, 1999; Amarante and Watanabe, 2009). However, this association has been marred with controversy. One of first studies to show an association between EBV and breast cancer was published in 1995 (Labrecque et al., 1995). Since then, several dozen papers have been published, some of which have reported an association between the virus and the pathogenesis of breast cancer, while others have failed to show this link [reviewed in (Amarante and Watanabe, 2009)]. These discrepancies between the different studies are probably due to the differing methodologies used for the detection of EBV, the histological types of tumors examined and the ethnic/geographical background of the cases studied. For example, using PCR-based techniques, a number of studies have reported a positive correlation between EBV and breast cancer, with approximately 50% of cases giving a positive signal (Labrecque et al., 1995; Bonnet et al., 1999; Fina et al., 2001; Murray et al., 2003; Tsai et al.,

Abbreviations: EBV, Epstein-Barr virus; EBERs, Epstein-Barr encoded RNAs; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; ISH, in situ hybridization; FFPE, formalin-fixed paraffin-embedded.

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2005; Preciado et al., 2005; Arbach et al., 2006; Fawzy et al., 2008). Moreover, a recent study by (Mazouni et al. 2011) reported the presence of EBV DNA in 33% of their cases and found that the virus was associated with aggressive forms of the disease. However, the fact that EBV is a ubiquitous virus present asymptomatically in over 90% of the world population, its mere detection in tumor tissue cannot be used to imply disease association. Indeed studies which have used the highly sensitive technique of Epstein-Barr encoded RNA-in situ hybridization (EBER-ISH) have tended to report negative correlation, even in cases which were EBV PCR positive (Glaser et al., 1998; Deshpande et al., 2002; Herrmann and Niedobitek, 2003; Murray et al., 2003; Thorne et al., 2005). Immunohistochemical studies which have reported the expression of EBV-encoded latent protein EBNA-1 in breast tissues, have also been questioned since the antibodies used to detect EBNA-1 have been shown to cross-react to other cellular proteins (Hennard et al., 2006; Murray, 2006). In this study we have employed EBER-in situ hybridization and immunohistochemistry (IHC) to address the following questions: Is EBV present in the malignant cells of breast cancer patients from the United Arab Emirates? Is there any correlation between the presence of EBV and the expression of estrogen, progesterone and HER2 expression and metastasis?

Methods

Patients

Formalin-fixed paraffin-embedded (FFPE) tissues from Emirati women diagnosed with breast cancer between May 2003 and December 2008 were retrieved from the Department of Pathology archives after receiving ethical approval from the Al Ain Medical District Human Research Ethics Committee (application number AAMD HREC 08/39). The following inclusion criteria were used: Emirati women (approximately 80% of the population of UAE comprises expatriates, hence all non-national women with breast cancer were excluded), histologically confirmed breast cancer, and sufficient material for analysis. The histology of all cases was reviewed independently by two consultant histopathologists. A total of 219 samples from 61 cases were finally included in the study. All, but 4 cases had multiple tissues (between 2–6, benign and malignant) that could be studied. Briefly, these consisted of (a) breast tissues: 174 samples (123 with histological evidence of malignancy, 4 benign, 47 tumor free); (b) lymph nodes: 45 samples (35 with evidence of metastasis and 10 free of malignancy). The mean age of our cases was 48 years (median 47, range 20–97 years).

Immunohistochemistry

For the detection of estrogen, progesterone and HER2 receptor expression in tumor cells, 5 μM sections were cut from FFPE blocks and processed for immunostaining using the Dako EnVision Kit (cat# KS5007) (Dako Ltd) according to standard protocols. The following primary monoclonal antibodies were used:

(a) For estrogen receptor: Clone SP1 (Cat.# RM-9101-S) (Lab Vision, CA, USA)
(b) For progesterone receptor: Clone 1A6 (Cat.# RM-9102-S) (Lab Vision, CA, USA)
(c) For HER2 receptor: Clone SP3 (Cat.# RM-9103) (Lab Vision, CA, USA)

Gill hematoxylin was applied as a counterstain and the sections mounted using DPX.

Scoring

ER and PR nuclear stains were categorized as positive or negative. A staining was considered positive when >10% of the cell nuclei were stained. HER2 expression was graded on a scale 0–3, where 0–1 was considered negative (absence or incomplete membrane staining); 2+ was considered equivocal (complete membrane staining but in less than 30% of the cells) and 3+ was considered positive (complete membrane staining in more than 30% of the cells).

EBER in situ hybridization

The presence of EBV in breast cancer tissues was determined by using a very sensitive and highly specific technique of EBER-ISH, essentially as described in detail in our previous publications (Khan et al., 1992; Khan, 2009). Briefly, 5-μm sections on sialinized slides were dewaxed and digested with 100 μg/ml proteinase K for 15 min at 37 °C. Sections were subsequently washed in water, dehydrated in ethanol and hybridized with digoxigenin-labeled EBER-1 and EBER-2 overnight at 42 °C. After stringency washes in 0.1x SSC at 55 °C, hybridized probes were detected using mouse anti-digoxin monoclonal antibody (Sigma, UK) and the avidin–biotin complex–peroxidase technique (ABC-Elite kit, Vector Laboratories, UK) using DAB as the chromogenic substrate. Sections were mounted in a permanent mount and examined. It should be noted that EBER-ISH was performed blindly without knowledge of the other assays or the histological status of the tissue samples. With each batch of 12 sections, a positive control (Hodgkin lymphoma known to be EBV positive, or EBV-infected B95-8 cell line) and a negative control (without EBER probes) was included.

Results

Histology

A total of 61 malignant breast cancer cases from Emirati women were available for study. On histological review, these cases were classified as follows: invasive ductal (47 cases), invasive lobular (1 case), ductal carcinoma in situ (11 cases), malignant phylloid tumor (1 case) and intracystic carcinoma (1 case). For statistical analysis, we grouped all cases into invasive ductal and others (Table 1). The mean age of our cases was 48 years (median: 47, range 20–97 years). Age distribution of our cases revealed that 59% of the cases were below the age of 50 years.

Immunohistochemistry

Of the 61 cases, sufficient material was available from 59 cases for immunostaining for ER, PR and HER2 expression. Overall, 54% of the cases showed strong nuclear staining for ER, 49% showed strong nuclear staining for PR and 36% showed strongly membrane staining

Table 1
Staining characteristics of Emirati breast cancer tissue.

Type of breast cancer	Number of cases	ER+	PR+	HER2+	$ER^-/PR^-/HER2^-$	EBER-ISH
ID (%)	47 (77)	25/46 (54)	23/46 (50)	14/46 (30)	11/46 (24)	21/47 (45)
Others (%)	14 (23)	7/13 (54)	6/13 (46)	7/13 (54)	1/13 (8)	8/14 (57)
Total (%)	61 (100)	32/59 (54)	29/59 (49)	21/59 (36)	12/59 (20)	29/61 (48)

Staining characteristics of 61 malignant carcinomas from Emirati women. Cases were grouped into two categories based on histology: Invasive ductal (ID) (47 cases) and others (14 cases, consisting of invasive lobular (1 case), ductal carcinoma in situ (11 cases), malignant phylloid tumor (1 case) and intracystic carcinoma (1 case). Cases were stained for estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and EBER-ISH. The staining for ER, PR and HER2 represent staining in malignant cells, while the EBER-ISH represents staining observed in non-malignant infiltrating lymphocytes. Malignant cells in all of 219 samples from the 61 cases were negative for EBV. Figures in parenthesis are percentages to the nearest whole number.

for HER2. 15% of the cases were weakly positive for HER2. 12/59 (20%) were triple negative. The results are summarized in Table 1.

EBER-in situ hybridization

EBER-ISH was performed on a total 219 samples from 61 cases. Of the 61 cases, 57 had had multiple tissues (2–6 samples/cases) that could be studied. Of the 219 samples tested, none had EBV in the malignant cells. However, occasional EBV-positive infiltrating lymphocytes were seen in 42/219 (19%) of the samples (Figs. 1a–d). These 42 samples were from 29 of 61 cases examined. Thus, 48% of the breast cancer cases in this series had one or more samples positive for EBV in lymphocytes. Analysis of ER, PR and HER2 expression in EBV lymphocyte positive and EBV negative cases did not reveal any correlation between the presence of EBV and the expression of any one of these receptors (Table 2). However, it appears that EBV positive cases were less likely to be triple negative compared to EBV negative cases (14% vs 26%). This is however, not statistically significant ($P = 0.272$). It is also note worthy that EBV-positive lymphocytes were more commonly seen in lymph nodes as compared to breast tissues [26/174 (15%) vs 16/45 (36%), chi-square $P = 0.002$] respectively.

Discussion

Breast cancer is a major global problem and a leading cause of morbidity and mortality (Ferlay et al., 2010). Thus, understanding the etiopathogenesis of this malignancy could lead to better treatment and preventative measures. Although numerous risk factors have been identified to be associated with the pathogenesis of some cases of

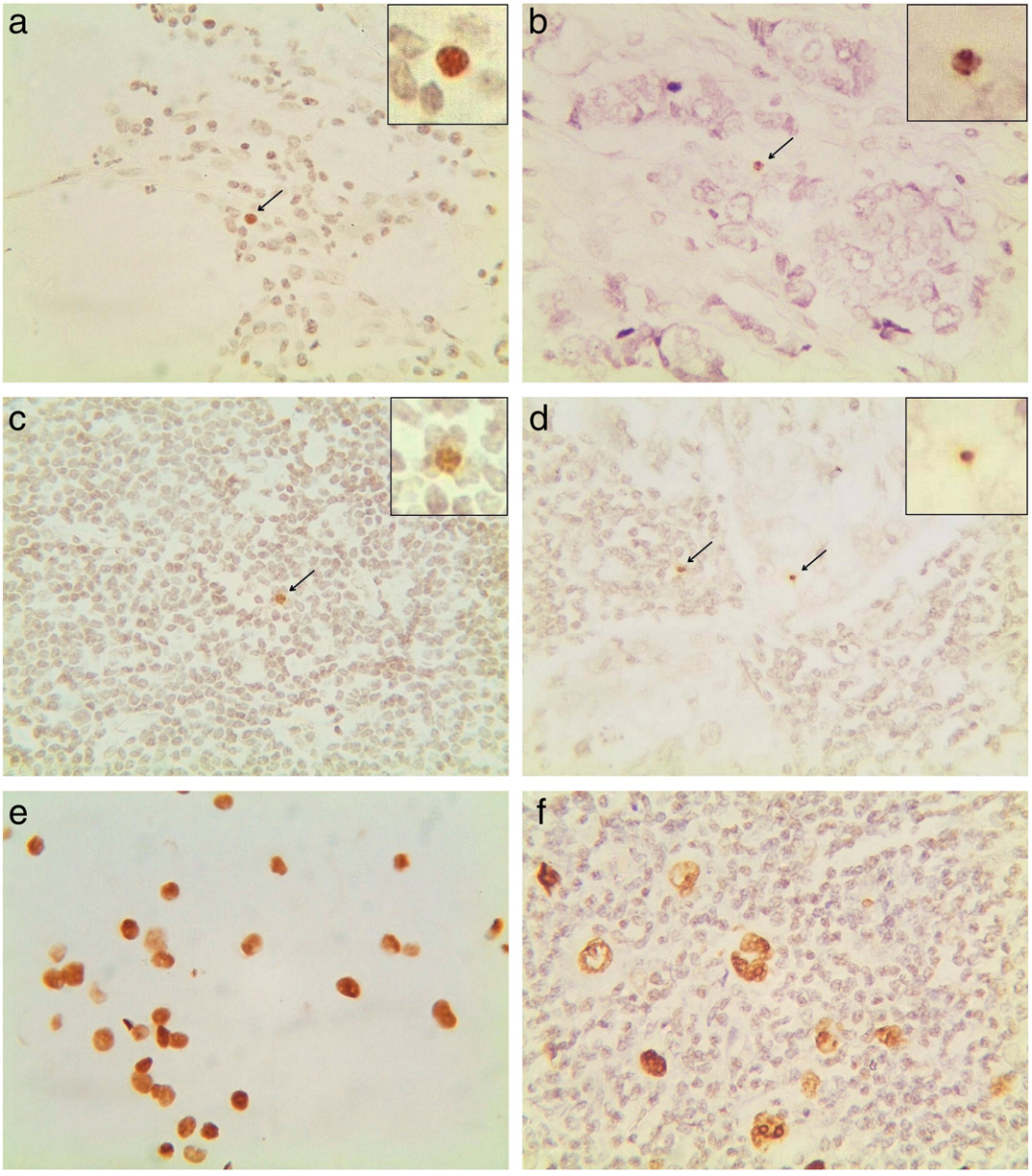

Fig. 1. Detection of EBV by EBER-in situ hybridization. EBER-ISH showing positive signal (brown) in an occasional infiltrating lymphocyte (arrow) in (a) breast tissue — reactive (b) breast tissue — invasive ductal carcinoma (c) lymph node — reactive (d) lymph node — metastatic ductal carcinoma. Figure (e) and (f) are EBV-positive controls, B95-8 cell line and Hodgkin lymphoma respectively. Strong nuclear signal typical of EBER-ISH staining pattern is evident in all images. In HL, only the malignant cells, the so called Hodgkin and Reed–Sternberg cells are positive for the virus, supporting the accepted view that EBV is etiologically associated with this malignancy. In contrast, the malignant cells in breast cancer showed no evidence of EBV presence in 219 samples from the 61 cases we examined (original magnification ×400).

Table 2
Staining characteristics of EBV-positive and EBV-negative cases.

EBER-ISH	Number of cases	ER+	PR+	HER2+	ER−/PR−/HER2−
EBV+ (%)	29 (48)	18/28 (64)	14/28 (50)	11/28 (39)	4/28 (14)
EBV− (%)	32 (52)	14/31 (45)	15/31 (48)	10/31 (32)	8/31 (26)
P values		0.337	0.990	0.956	0.272
Total (%)	61 (100)	32/59 (54)	29/59 (49)	21/59 (36)	12/59 (20)

Receptor staining status in relation to EBV-positivity in lymphocytes. A total of 61 breast cancer cases were studied. EBV was found in infiltrating lymphocyte in 29/61 cases (48%). EBV was not found in malignant cells in any case. Of the 29 EBV-positive cases and 32 EBV-negative cases, 28 and 31 respectively were available for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) staining. Triple negative cases are also indicated. Figures in parenthesis are percentages to the nearest whole number.

breast cancer, in the vast majority of cases no risk factor has been conclusively identified. A number of studies have reported the presence of EBV in a subset of breast tumors, thereby implying a role for this virus in the pathogenesis of breast cancer (Xue et al., 2003; Preciado et al., 2005; Arbach et al., 2006; Fawzy et al., 2008; Joshi et al., 2009). However, negative results have also been reported (Chu et al., 2001; Deshpande et al., 2002; Perrigoue et al., 2005; Cox et al., 2010; Kadivar et al., 2011), making the association of EBV with breast cancer a very controversial topic. The differences are probably due to the different methods used for the detection of EBV in breast tissues (Magrath and Bhatia, 1999). Here, we report our findings from a large series of Emirati breast cancer cases using a highly sensitive and specific technique of EBER-in situ hybridization (Khan, 2009). This technique involves targeting two non-protein coding RNAs produced in all EBV infected cells. These RNAs, referred to as Epstein-Barr encoded RNAs (EBERs) are by far the most abundant gene transcripts in infected cells (10^7 copies per cell). These small RNAs, 166 and 172 nucleotides are located in the nucleus where they are associated with cellular proteins (Arrand and Rymo, 1982; Glickman et al., 1988). The principle of EBER-in situ hybridization is to target EBERs as a means of detecting EBV in histological material using digoxigenin-labeled complementary oligonucleotides (Khan et al., 1992). Using this approach, we did not find any evidence for an etiological role for EBV in the pathogenesis of Emirati breast cancers. The virus was however found in occasional infiltrating lymphocytes in 48% of the cases studied, but the malignant cells were clearly negative. Since EBV is a B-cell tropic virus, and the infected cells in our cases had typical lymphoid morphology, it is likely that these EBV-positive cells were indeed B-lymphocytes. However, we cannot state this for certainty without double staining i.e. EBER-ISH and immunohistochemistry for B-cell markers. Dual staining however is a very challenging technique and in cases in which the infected cells are less than a dozen, like in our series, double staining would be very difficult to achieve. Furthermore, considering that most of the world population is EBV-seropositive and the virus is known to establish life-long latency in B-lymphocytes, finding the virus in occasional lymphocyte is not surprising. We have previously shown that the frequency of EBV-infected B-cells in healthy seropositive individuals is in the range of 1–50 cell per 10^6 B-cells and that this frequency is relatively stable overtime in any given individual (Khan et al., 1996). Thus, the finding of occasional EBV-infected lymphocyte in breast cancer patients is most likely a reflection of what is seen in healthy individuals. Furthermore, the fact that EBV-infected lymphocytes were more commonly seen in lymph nodes (36% positive) compared to breast tissues (15% positive), supports this notion. These observations could also explain why some studies which have used PCR-based approaches have concluded that EBV is etiologically associated in the pathogenesis of breast cancer.

Although our findings suggest that EBV is not directly involved in the pathogenesis of breast cancer, we cannot rule out an indirect role via induction of inflammation. Indeed, there is now a substantial body of evidence supporting the notion that chronic inflammation plays an important role in the pathogenesis of a number of human malignancies (Mantovani et al., 2008; Grivennikov et al., 2010). In this context, EBV has been shown to induce the activation of a number of inflammatory factors which can contribute to tumor cell proliferation, progression and inhibition of apoptosis (Khan, 2006; Hannigan et al., 2011). Furthermore, pro-inflammatory regulators such as NFκB and STAT3 are thought to play a central role in many of these processes (Pikarsky et al., 2004; Hannigan et al., 2011). Future studies aimed at examining non-malignant inflammatory conditions and the pattern and distribution of EBV infected cells in such tissues may shed light on the relationship, if any, between EBV, inflammation and cancer.

UAE is a very cosmopolitan country with over 80% of the population being expatriates. All the cases included in this study were selected from Emirati nationals. The mean age of our cases was 48 years (median: 47, range 20–97 years). Age distribution of our cases revealed that 59% of the cases were below the age of 50 years. This figure is higher than what has been reported in many Western countries where majority of breast cancer cases occur in women older than 50 (Anderson et al., 2006; Chun et al., 2008). The few studies that have examined age-standardized incidence rates of breast cancer in Arab women have also reported a younger age at presentation compared to industrialized nations (El Saghir et al., 2002, 2007; Salim et al., 2009; Hemminki et al., 2011). The reasons for this disparity are not known. Interestingly, Arab women migrating to industrialized countries maintain an earlier mean age at diagnosis compared to their matched controls (Hemminki et al., 2011). Some studies have also reported that younger age and ethnicity are associated with a more aggressive form of breast cancer, the so called triple negative breast cancer (ER−/PR−/HER2−) (Carey et al., 2006; Podo et al., 2010; Parise et al., 2010). In this study we found 20% of the cases to be triple negative. The mean age of the triple negative cases was 47 years which was not significantly different from the non-triple negative cases (mean age 49 years).

Conflict of interest

We declare that we have no conflict of interest.

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