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RESEARCH

Epstein-Barr Virus and Breast Cancer: Lack of Evidence for an Association in Iranian Women

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Abstract Controversies regarding the role of Epstein-Barr virus (EBV) in breast cancer and lack of published literature in this regard in Iran, prompted us to assess EBV presence in 100 breast carcinoma and 42 control biopsies obtained from Iranian women. Breast carcinoma cases were comprised of 81 invasive ductal carcinoma NOS, 9 invasive lobular carcinoma, 1 apocrine carcinoma, 2 cribriform carcinoma, 2 papillary carcinoma and 5 mucinous carcinoma. Control biopsies consisted of 13 fibroadenoma, 9 benign epithelial proliferation (adenosis and sclerosing adenosis), 9 usual ductal hyperplasia, 4 atypical ductal hyperplasia, 4 non-proliferative fibrocystic changes and 3 normal breast tissue. To identify EBV-infected cells we applied immunohistochemical analysis, using monoclonal antibody against Epstein-Barr virus-encoded nuclear antigen 2 (EBNA-2) and latent membrane protein 1 (LMP-1). Further, polymerase chain reaction (PCR) was used to amplify EBV DNA, with primers that cover the EBV encoded RNA (EBER) and BamHIW regions. EBNA-2 and LMP-1 immunohistochemistry were negative in all breast cancer and control specimens. Using PCR, none of the 100 breast cancer samples or the 42 control specimens showed detectable EBV DNA. These results indicate that EBV may not play a significant role in the etiology of breast cancer in Iranian women.

Keywords Epstein–Barr virus (EBV) · Breast carcinoma · Iran · Immunohistochemistry (IHC) · Polymerase chain reaction (PCR)

Abbreviations

BZLF1	Immediate-early transcriptional activator
BamHIW regions	Internal repetitive region in Epstein-Barr virus genome
CS1-4	Monoclonal antibody for LMP1
EBER	EBV encoded RNA
EBNA-2	Epstein-Barr virus encoded Nuclear Antigen 2
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
IHC	Immunohistochemistry
LMP-1	Latent Membrane Protein 1
NPC	nasopharyngeal carcinoma
PE2	Monoclonal antibody for EBNA2
PCR	Polymerase chain reaction

Introduction

Breast cancer is one of the most common cancers among women worldwide. Although it is an epidemiologically significant global health problem with approximately 1,000,000 new cases diagnosed annually, identified risk factors are present in only about half of the cases [1, 2].

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There has been an increasing interest in investigating the possible role of viral infections in the breast cancer in recent years and Epstein-Barr virus (EBV) has been one of the candidate viruses [3].

In 1995, Labrecque et al. reported finding EBV in 21% of a series of 91 breast cancers [1]. Likewise, in 2008, Fawzy et al. showed EBV association with some invasive breast cancers in Egyptian females [4]. Further, in 2009, Joshi et al. reported expression of Epstein-Barr virus nuclear antigen 1(EBNA-1) in a significant proportion of breast cancer specimens in rural Indian women [5].

In contrast to the above findings, data from the other studies question this association. In 2005 in Turkey, Kallkan et al. demonstrated no etiological role for EBV in breast cancer [6]. Similarly, in 2010 in New Zealand, Cox et al. reported no association between changes in EBV IgG antibody and the risk of breast cancer [7].

In the view of the controversial results and lack of the published data regarding this issue in Iran, we aimed to assess EBV presence in the breast carcinoma specimens from a series of Iranian patients, by using polymerase change reaction (PCR) to amplify EBV encoded RNA (EBER) and BamHIW regions; and by applying immunohistochemistry (IHC) for detection of the Epstein-Barr virus encoded Nuclear Antigen 2 (EBNA-2) and Latent Membrane Protein 1 (LMP-1).

Material and Methods

Formalin-fixed, paraffin-embedded tissue blocks from 100 invasive breast carcinomas were randomly retrieved from the Department of Pathology at Atieh General Hospital, Tehran, Iran. These included 81 invasive ductal carcinoma NOS, 9 invasive lobular carcinoma, 1 apocrine carcinoma, 2 cribriform carcinoma, 2 papillary carcinoma, and 5 mucinous carcinoma. All patients were women who ranged in age from 25 to 90 (49.07±12.12). The study controls included biopsy specimens of 13 fibroadenoma, 9 benign epithelial proliferation (adenosis and sclerosing adenosis), 9 usual ductal hyperplasia, 4 atypical ductal hyperplasia, 4 non-proliferative fibrocystic changes, and 3 normal breast tissue from women whose age range was 19–54 years (36.5±10.33). All patients and controls were born and raised in Iran, with comparable early exposure to EBV.

Immunostaining was used to localize and quantify EBNA-2 and LMP-1 expression in tumor cells on formalin-fixed, paraffin-embedded tissue sections using monoclonal antibody PE2 (Dako, Danmark) against EBNA-2 and CS1-4 (Dako, Danmark) against LMP-1. Paraffin sections were deparaffinized and pretreated in 10 mM Tris and 1 mM EDTA solution (PH 9.5) for 5 min at 500 W in a microwave oven. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. Immunohistochemical detection of monoclonal antibody was performed with a streptavidin-biotin complex peroxidase detection system according to manufacturer's instruction. Hematoxyline was used for counterstaining. Hodgkin's lymphoma and lymphoma in immune deficient patients were used as a positive control for LMP-1 and EBNA-2, respectively.

PCR amplification was performed on paraffin sections of all cases. To confirm EBV presence, PCR was performed by amplifying EBV DNA sequences encoding for EBERs as described by Bonnet et al. [8]; and for the repetitive region IR1 in the BamHIW fragment of the EBV genome as described by Saito et al. [9]. For this purpose, five 10 micron paraffin section from each case was dewaxed in xylene for 30 min at room temperature, rehydrated through graded ethanol (30 min each in 100% and 70% ethanol) and air dried. Tissues were incubated in 500 ml buffer (1 M Tris-HCL [pH 8.3], EDTA 0.5 M, SDS 10%) and 0.2 mg/ml proteinase K overnight at 56 C, followed by heat inactivation of proteinase K.

Five microliter aliquots of DNA extracts were subjected to PCR: 5 min denaturation at 94 C, 10 cycles of 20 s at 94 C, 20 s at 58.3 C and 30 s at 72 C followed by 25 cycles of 20 s at 94, 20 s at 53.3 C and 30 s at 72 C, completed by 150 s at 72 C. The reaction volume was 25 μl containing 3 μl DNA, 1Xpolymerase buffer, 1.5 mM MgCl2, 200 mM dNTP, 1 U Taq-polymerase and 400 nM of each primer mix (CCCTAGTGGTTTCGGACACACA and ACTTG CAAATGCTCTAGGCG) that covered EBV encoded RNA (EBER) and (CCAGAGGTAAGTGGACTT AND GACCGGTGCCTTCTTAGG) which covered EBV BamHIW internal repetitive fragment. The primers resulted in the amplification of 108 bp of EBV EBER and 122 bp fragment of the EBV BamHIW internal repetitive fragment. In a separate reaction tube, PCR was performed to amplify a 563 base pair fragment of B-globin gene as a control to monitor the amplification ability of a single-copy gene. Amplified DNA was subjected to electrophoresis on a %1.5 agarose gels with ethidium bromide.

IHC and PCR results of the breast carcinoma cases and control specimens were recorded in tables and compared afterwards. The ethics committee of the institution approved the study.

Results

None of the breast carcinoma or benign lesions specimens which were subjected to IHC showed Nuclear staining which is typical for EBNA2 and none of them showed membrane staining which is typical for LMP1 expression.

We did not detect amplification fragments of either 108 bp from EBER regions or 122 bp from the BamHIW

by PCR in any of the breast carcinoma biopsies. Neither EBER PCR nor BamHIW PCR detected any amplification product for EBV in biopsy specimens of 13 fibroadenoma, 9 benign epithelial proliferation (adenosis and sclerosing adenosis), 9 usual ductal hyperplasia, 4 atypical ductal hyperplasia, 4 non-proliferative fibrocystic changes, and 3 normal breast tissue.

Discussion

Among the 15 studies using PCR to find EBV in breast tumors, the virus was found in 0–66% of the specimens. Prevalence was highest when PCR targeted the reiterated *Bam*H1W sequence and the EBER [8, 10–14]. Using DNA PCR to detect EBER-1 and EBER-2, Labrecque et al. found 19 of 91(21%) British breast cancer cases to be EBV-positive. All normal or benign breast tumors in this study were negative by PCR [1]. Luqmani et al. also found 15 of 28 (42%) British breast cancer cases to be positive using PCR [11]. Bonnet et al. found 51 of 100 (51%) French breast carcinoma cases to be EBV-positive for EBER-2 and LMP-2[8].

In contrast, other researchers did not detect EBV in 52 infiltrating ductal carcinoma with medullary feature by PCR [15]. Another study reporting 10 cases examined by PCR, in situ hybridization, and IHC, failed to show the presence of EBV in breast carcinoma specimens [16].

By using EBER in situ hybridization and EBNA-2 and LMP-1 IHC, Chu et al. evaluated breast carcinoma samples in a Taiwanese population with high incidence of EBV-associated nasopharyngeal carcinoma (NPC) and demonstrated no EBV in 60 cases [17]. In 2005, in Turkey, Kallkan et al. found no etiologic role for EBV in breast cancer after detecting equal EBV DNA in both breast cancer specimens (13/57=23%) and normal breast tissues (19/55=35%), using PCR [6]. Using IHC analysis, in 2008 Fawzy et al. found positive stain for EBV nuclear antigen 1 (EBNA-1) in 10/40 (25%) of breast cancers specimens in Egyptian women. PCR detected EBV-DNA in 8/10 of those EBNA-1 positive breast cancer specimens [4]. Likewise in 2009, Joshi et al. demonstrated positive EBNA-1 stain in 28/51(54.9%) of breast cancer specimens from rural Indian women by using IHC [5].

In a recent study using laser capture microdissection combined with real-time quantitative PCR, Arbach et al. detected EBV genomes in about 50% of breast cancer specimens. They found a highly variable viral load from one tumor to other. Furthermore, EBV genomes were heterogeneously dispersed in morphologically identical tumor cells; some clusters of isolated tumor cells were negative for EBV-DNA genome, while other tumor cells isolated from the same specimen showed relatively high genome numbers [18]. In another study, using real-time quantitative PCR, Serene Rerkins et al. showed that a high percentage of breast cancer biopsies (46%) were EBV positive. Surprisingly, the EBV positive tumor biopsies contained an extremely low viral load [19].

Although EBERs have been a constant feature of all known EBV-related cancers, they are not found in all NPCs or in some Burkitt's and Hodgkin lymphomas that are LMP1-positive or positive by PCR. In breast cancer, EBERs may be expressed in less number than other malignancies or breast carcinoma may maintain a previously unrecognized form of EBVinfection characterized by EBER down-regulation, as recently suggested for liveradenocarcinomas [20].

Based on these considerations, we looked for the presence of both theEBER and BamHIW regions of EBV DNA and the expression of other viral gene products likeEBNA2 and LMP-1.

However, we did not detect amplification fragments of either 108 bp from the EBER regions or 122 bp from the BamHIW region of EBV by PCR in any of the breast carcinoma or benign breast lesions. Using IHC, our study revealed negative result for LMP-1 and EBNA2. IHC targeting LMP1 is a sensitive and widely employed assay but is limited by the fact that LMP1 is absent in some otherwise EBV-related neoplasm [20]. Six IHC studies targeting LMP1 had negative results for breast carcinoma [11, 16, 17, 21–23]. IHC targeting EBNA2, LMP2a, and the BZLF1 viral replication factor yielded negative results [23].Geographic variation of EBV infection has been observed in many EBV-associated neoplasms, including Hodgkin's disease, NPC, and Burkitt's lymphoma. For example, the incidence of EBV associated Hodgkin's lymphoma is higher in Latin America than in developed countries and the incidence of EBV infection in African Burkitt's lymphoma is much higher than in other parts of the world [24]. In breast carcinoma, before the year 2000, the studies reporting the association between EBV and breast carcinoma were mostly from European countries like England and France; reports from Japanese and Taiwanese patients, did not detect EBV by PCR, insitu hybridization, and IHC[1, 8, 11, 17]. However, new reports from Egypt (2008) and India (2009) demonstrated the association between EBV and breast cancer while other reports from Turkey (2005) and New Zealand (2010) disapproved this association. In the current study, none of breast cancer specimens were positive for EBV by PCR and IHC. The reasons for these discrepancies are not clear, but racial or geographical factors may play a role.

Our findings suggest that EBV may not play a significant role in the pathogenesis of breast cancer in Iranian patients. However, with the complexity existing in the connection of EBV with other cancers, the presence of

technical problems in detecting EBV in breast cancer, the possibility of yet undiscovered pathways of EBV pathogenesis in epithelial neoplasm and the inadequate epidemiological issue up to now, no definite judgment regarding the association between EBV and breast cancer can be made. Considering the apparently low prevalence of EBV in breast cancer, new studies with greater sample size may be more informative. Recent advances in laboratory methodologies such as real time PCR with laser capture microdissection should help overcome the challenges of EBV detection in breast cancers.

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