Histopathological and Molecular Studies For An association Between Breast Cancer and Epstein-Barr virus In Iraqi Population.


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ABSTRACT

The majority type of cancer between women in worldwide is breast cancer (BC). The relationship between Epstein-Barr virus (EBV) and breast carcinogenesis is still controversial. The goal of the present research was to explain the involvement of EBV and its role in development of BC in both females and males in Karbala population. In the present study, sixty two subjects were divided into four groups as the following: (group I: 32 women infected with BC, group II: 10 intact women as a control, group III: 10 men infected with BC and group VI: 10 intact men as a control) and evaluated using paraffin embedded tissues. In this survey study, higher percentage (46.875%) of affected females with BC were found at age (41-50) years and (25%) of affected males with BC were found at age (51-60) years. DNA of EBV in pathological samples was detected by Polymerase chain reaction (PCR) as well as PR and ER markers was analyzed via immunohistochemistry techniques, in the result, there was no significant difference between the grade of EBV tumor with the age groups between females and males. Furthermore, there were no significant differences at (P>0.05) in females in which 20/29 (69%) of females have been affected with IDC with positive EBV-DNA in their investigated samples, while 9/29 (31%) of females were negative, OR (1.11) IC (0.088-13.89). Moreover, 2/3 (66.7%) of females have been affected with ILC with positive EBV-DNA in their investigated samples, while 1/3 (33.3%) of females were negative. On the other hand, 8/10 (80%) of the Male patients were affected with IDC and they were positive for EBV-DNA in their investigated samples, while 2/10 (20%) of males were negative, and there was no significant differences in ages between affected patients.

Keywords: EBV, Breast cancer, carcinogenesis, PCR


INTRODUCTION

Breast cancer is the most commonly detected malignancy of females, it is the second cause of death in the worldwide which develop in breast tissues that include changes in the morphology of breast, skin dimpling, lump, fluid pending from nipple and red scaly patches of skin (Zekri et al., 2012 and Reza et al., 2015). Breast cancer is more common in the developed populations and is 100 times more in females than in males (Ito, and Matsuo, 2016). Observation of the increased risk factors of breast cancer are positive family histories, age, the primary pregnancy following 25 years old, menarche, delayed menopause, nulliparity, long-standing utilization of exogenous estrogens, obesity following menopause, as well as encountering ionizing ray (Kumaret et al., 2014). Other factors that important in the mechanism of
increased risk factors are estrogen receptors, estrogen levels, and the adipokines leptin and adiponectin (Hsu et al., 2010). The International Agency for Research on Cancer (IARC) found that biological factors result in 18-20% of different types of malignancies; Viruses have been recorded as an important reason for breast cancers growth such as Epstein-Barr virus, Polyoma virus and Mouse mammary tumor virus (MMTV) (Ban and Godellas, 2014). Some authors were detected Epstein-Barr virus (EBV) in the neoplastic tissues of breast cancer (Joshi et al., 2009), this type of virus was first time documented by two investigators, Barr and Epstein in 1964, the most important method for transmitting EBV was through directional contact and saliva, whereas infectivity via transplantation, transfusion, and placenta is also potential (Henry, 2001). Many diseases caused by (EBV) virus such as Burkett’s lymphoma, multiple sclerosis (MS), nasopharyngeal carcinoma (NPC), Non-Hodgkin’s lymphoma, Hodgkin’s lymphoma, and gastric tumor (Brooks et al., 1992; Lindhout et al., 1994; Morshed et al., 2002 and Gandhi et al., 2004). The relationship between EBV and breast cancer is very important not for understanding the causes of breast cancer only, but also for early diagnosis, treatment and protection against the tumor (Mant et al., 2004). In the present study, presence of EBV in paraffin-embedded malignant tissue samples of BC (ILC, IDC) in Iraqi women and men patients were diagnosed by immunohistochemistry markers (ER, PR, HER2) and PCR assays for demonstrating the critical association of EBV with breast cancer.

**Materials and Methods**

**Subjects**

Pathological specimens from Breast Cancer (BC) patients were taken by Paraffin embedded blocks, collected from Al-Hussein Teaching hospital and private Pathology Laboratories in Karbala province-Iraq during the period from January to April 2018. A total of 62 specimens were divided into four groups as the following (group I: 32 women patients, group II: 10 intact women as control, group III: 10 men patients, group IV: 10 intact men as control). In the present study detection of Breast Cancer was based on the breast biopsies according to the clinical criteria of affected patients (females and males) with Breast Cancer.

**Preparation of Deparaffinated Samples**

A total of Sixty two breast samples were Paraffin embedded blocks for infected and normal breast tissues were cut up to 25 mg in each microcentrifuge tube. 1 ml of xylene was added to each sample and mixed well by vortex, then, incubated for 30 minutes at room temperature, centrifuged at 16000 rpm for 3 minutes, supernatant was discarded by pipetting, and 1 ml of ethanol was supplemented to each sample, mixed lightly by vortex, centrifuged at 16000 rpm for 3 min., supernatant was discarded by pipetting and twice recurring of this stage, the sample was incubated for evaporated ethanol at 37 °C., finally, pathological tissue samples were grinded by micropustles and prepared for tissue digestion according to technique of (Kokkat et al., 2013).

**Tissues digestion and DNA Extraction**

In the present study each pathological tissue sample in 1.5 microcentrifuge tube was digested by adding of Tissue Lysis Buffer which consisted of (200 µl FATG1, 20 µl proteinase K), incubated at 60°C for 10 min. until completely lysis of tissues, then, (200 µl FATG2, 200 µl ethanol) was supplemented to each tube, incubated at 70°C for 10 min, then, 400 µl W1 buffer was supplemented, then, 750 µl washing buffer was supplemented to FATG, then, 100 µl of elution buffer (PH 7.5-9.0) was supplemented to the membrane of FATG, finally the contents were centrifuged at 16000 rpm for 2 min. for DNA elution. DNA isolation of all pathological samples was achieved by using tissue protocol (Flavorgene/Korea) kit, in proportion to the producer information, extracted DNA aliquots were measured with Q5000 UV-Vis Spectrophotometer, 20-25 nanogram /microliter of extracted DNA aliquots were used for molecular detection of DNA of EBV.
Detection of EBV by Polymerase chain reaction Technique

The following Primers sets in table.1 was used for amplifying DNA of all breast cancer and control samples according to (Khabaz, 2013).

Table1: Primers sequences used for amplifying DNA of EBV

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotides sequences</th>
<th>G-C %</th>
<th>product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5-CTAGCGACTCTGCTGGAAAT-3</td>
<td>53%</td>
<td>337 bp.</td>
</tr>
<tr>
<td>Reverse</td>
<td>5-GAGTGTGTGCCAGTTAAGGT-3</td>
<td>50%</td>
<td></td>
</tr>
</tbody>
</table>

All malignant and ordinary DNA samples were depended for detecting EBV by PCR technique was performed according to the Amplification cycle conditions which were consisted of an initial step of DNA denaturation at 96ºC for 2 min., subsequent by 35 cycles of denaturation at 96ºC for 30 seconds, and annealing at 55ºC for 1min., extension by 72ºC for 2min. and a final extension by 72ºC for 10 min..DNA products of PCR technique were electrophoresed in 2% agarose gel stained with 0.5 μg/ml of ethidium bromide and visualized with UV light and recorded in Gel documentation system according to (Khabaz, 2013).

Immunohistochemistry (IHC) Technique

Principle

Ultra vision quantum recognition system mouse or rabbit bound to an antigen in tissue section. The specific antibody was applied by a universal secondary antibody formulation conjugated to an enzyme labeled polymer that recognizes mouse and rabbit antibody. The polymer complex was then visualized with a suitable substrate \ chromogen according to (Stacchiotti et al., 2013). All steps of immunohistochemistry technique were performed at room temperature including deparaffinization, rehydration of tissue sections and incubation of tissues in an appropriate pretreatment or digestive enzyme, then, incubation of slides in ultravision hydrogen peroxide blocks for 10 min., then, application of ultravision protein blocks and incubation for 5 min., application of primary antibody (ER, PR and HER2) and incubation for 10min. and washing the slides with washing buffer, then, Primary Antibody Amplifier Quantowas applied and incubated for 10min.,and 30 µl DAB Quantochromogen was supplemented to 1 ml of DAB substrate, mixed by swirling and applied to tissues and incubated for 5 min. followed by washing by DI water and applied of counter stain and cover slip using permanent media (Shiet et al., 1999).

Statistical analysis

Chi-square test (Fisher’s exact test) was conducted for assessment the association between the presences of DNA virus genome of EBV in breast cancers using (SAS program, V12).

Results

In the present study, 32 females were infected with breast cancer (30 IDC, 2ILC) and 10 normal breasts tissues as well as 10 male patients with breast cancer and 10 normal breasts tissues were infected with the breast cancer, table (2) demonstrated high percentage (40%)of male patients with breast cancer was recorded at the ages from 51-60 years, while the high percentage (45.5%) in females patients occur at the ages from 41-50 years while the lowest percentage (3%) of breast cancer was appeared in females at ages from 20-30 years and there were no significant differences in genderat (P>0.05)appeared between male and females patients. Moreover, table (3) revealed significant differences between males and females in the grade of breast cancer. Furthermore, table (4) revealed no statistical significance in relationship between EBV and grade of tumor in both genders of male patients and females. Also, table (4)
revealed 20/29 (69%) positive females and 9/29 (31%) negative females infected as intra ductile cancer (IDC) with EBV, while 2/3 (66.7%) positive females and 1/3 (33.3%) negative females infected as intra lobular cancer (ILC) with EBV. The female patients with positive breast cancer due to EBV were more sensitive to intra ductile cancer 1.11 times more often than females with negative EBV, odd ratio (OR: 1.11) and confidence interval (CI) (0.088-13.89). On the other hand, 8/10 (80%) positive males patients were infected as intra ductile cancer (IDC) with EBV and only 2/10 (20%) negative males were infected as intra ductile cancer (IDC) with EBV, table (4). Analysis of (DNA products of PCR technique for infected females with BC with EBV.) by agarose gel electrophoresis demonstrated in figures (1,2).

Table (1): revealed the relationship between ages and genders of patients with the breast cancer.

<table>
<thead>
<tr>
<th>No. of male</th>
<th>%</th>
<th>No. of female</th>
<th>%</th>
<th>Age</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>30</td>
<td>2</td>
<td>20</td>
<td>15-20</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>5</td>
<td>50</td>
<td>21-30</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>31-40</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>40-50</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>3</td>
<td>30</td>
<td>50-60</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>60-70</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>10</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

X²=2.55  
P= 0.63

Table (2): revealed the relationship between ages and genders of a control group.

<table>
<thead>
<tr>
<th>No. of males</th>
<th>%</th>
<th>No. of females</th>
<th>%</th>
<th>Age</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3.125</td>
<td>20-30</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>3</td>
<td>9.375</td>
<td>31-40</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>15</td>
<td>46.875</td>
<td>41-50</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>8</td>
<td>25</td>
<td>51-60</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>5</td>
<td>15.625</td>
<td>61-70</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>32</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

X²=2.55  
P= 0.63

Table (3): revealed the relationship between the grades of infection of Breast Cancer patients with their gender.

<table>
<thead>
<tr>
<th>No. of male</th>
<th>%</th>
<th>No. of female</th>
<th>%</th>
<th>Grade of tumor</th>
<th>Statistical analysis</th>
</tr>
</thead>
</table>
| 3           | 30 | 4             | 12.5| Grade 1        | X²= 2.323  
P value = 0.313 |
| 5           | 50 | 15            | 46.9| Grade 2        |                      |
| 2           | 20 | 13            | 40.6| Grade 3        |                      |
Table (4): demonstrated the relationship between types of cancer related to EBV infection.

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>N. of patients</th>
<th>Positive EBV</th>
<th>Negative EBV</th>
<th>OR (95% CI)</th>
<th>Pvalue</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDC (Reference)</td>
<td>29</td>
<td>20 (69%)</td>
<td>9 (31%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ILC</td>
<td>3</td>
<td>2 (66.7%)</td>
<td>1 (33.3%)</td>
<td>1.11 (0.088-13.89)</td>
<td>0.93</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDC (Reference)</td>
<td>10</td>
<td>8 (80%)</td>
<td>2 (20%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ILC</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>42</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure (1): Agarose gel electrophoresis for DNA products of PCR for infected females with BC with EBV. Lane 1: DNA ladder -100 bp., The Lanes (2-7): DNA products with 337bp.
Figure (2): Agarose gel electrophoresis for DNA products of PCR for infected males with BC with EBV and control. Lane 1: DNA ladder -100 bp., Lane 2 represents DNA samples of controls male Lanes 3-5: DNA samples of infected males with EBV. Lanes 6-7: DNA samples of control females.

Fig. (1) Normal female breast tissue, H&E stain x200

Fig. (2) Normal male breast tissue, H&E stain x100
Fig. (3) Invasive lobular carcinoma, H&E stain x200

Fig. (4) Invasive lobular carcinoma progesterone receptors positive x100.

Fig. (5) Invasive lobular carcinoma, H&E stain x100

Fig. (6) Invasive lobular carcinoma, estrogen receptors positive x400.
Fig. (7) Invasive ductal carcinoma, progesterone receptors positive, x100

Fig. (8) Invasive ductal carcinoma, HER2-neu positive, score 3, x400

Fig. (9) Invasive ductal carcinoma, HER2-neu positive, scores 3, x400.

Fig. (10) Invasive ductal carcinoma, H&E stain x200.
Many previous studies have been involved for decades by the hypothesis that human breast cancer may be caused by a virus (Hsu et al., 2010). EBV may interfere with cell development in more than one pathway, infection with EBV is an important stage in tumor development and danger factors of epidemiology, it is stated in about 50% of breast cancers by divers studies (Zekri et al., 2012). Current study have revealed that EBV present in the breast tissues for 20/29 (68.9%) females with BC type (IDC) and 2/3 (66.66%) females for (ILC) from total 32 women as well as 8/10 (80%) males with BC type (IDC) from total 10 men of patients affected with breast cancers. Several observations have been found various relationships between breast cancers with EBV: (a) EBV was found in the tissue of breast, and then it was detected in the milk of the breast for some women (Junker et al., 1991). (b) Transfection of EBV DNA promotes development of milk production cells in human mammary gland (Kumar et al., 2014). (c) Presence of some lymphomas in the breast tissues associated with EBV (Giardini et al., 1992 and Abhyankar et al., 1998). (d) Breast tumor has epidemiological similarities to young-adult Hodgkin’s lymphoma, although evidence for breast tumor involves timing of prime EBV infection sooner than viral oncogenesis (Yasui et al., 2001). (e) Benign breast cancers in immunosuppressed females was identified with EBV (Kleer et al., 2002). (f) in vitro, epithelial cells of mammary gland can be affected via direct attachment with EBV-bearing lymphoblastoid cell lines (Speck and Longnecker, 2000). This finding was in agreement with related previous studies by two scientists of Egypt (Mohamed et al., 2007 and Fawzy et al., 2008) who have reported that EBV infections were found in 35.3% and in 25% of invasive breast tumors respectively. The present study revealed the critical result which was agreed with similar findings found by (Preciado, 2003 and Perkins et al., 2006) where infection with EBV has been detected in 10–50% according to PCR and histochemical techniques have recorded an evidence of existence of DNA or proteins of EBV within the breast tumor cells which proposes a pathogenic function of
EBV. Furthermore, (Mazouni et al., 2011) found 65 of 196 (33%) of cases of breast cancer had DNA of EBV via RT-PCR. Also, (Mohamed et al., 2007) suggested a potential connection between EBV and breast cancer when it was detected in 35.3% of samples. As well as (Zekri et al., 2012) reported EBV-DNA was found in 90 Egyptian and Iraqi women affected with breast cancers and EBV was detected in 45% (Egyptian) and 28% (Iraqi) positive females respectively. On the other hand, Present study have been demonstrated marked variation in prevalence of EBV marker by using the cross-reactivity immunostains in immunohistochemistry technique, these observations were closely related with the findings of (Zekri et al., 2012) that displayed positive cytoplasmic immunostaining for LMP-, positive membranous immunostaining for CD21 and positive nuclear in situ hybridization with EBER-1 for (IDC). Finally, the present study was in agreement with the findings that have been concluded by (Brink et al., 2000 and Ahmed and Yussif, 2016) who were demonstrated positive immunohistochemical staining for EBV breast cancer type (IDC).

CONCLUSIONS
Within the limitations of this investigation, we have identified EBV have a direct role in the pathogenesis of an important clinical cases of breast cancer both IDC and ILC types in females and males of Iraqi population, and there was no significant differences in ages between affected patients. These findings were achieved with the using of molecular investigation by PCR as well as immunohistochemistry techniques for confirming existence of DNA of EBV in the breast tissues of affected males and females with breast cancers.

ACKNOWLEDGEMENTS
We would like to thank all participants who have helped in submission of required blood and breast tissue samples. Moreover, we would also like to thank all the staff of departments of physiology and Microbiology in the college of veterinary medicine in the University of Kerbala for their supports for achieving these findings.

REFERENCES


