Original Article

Evaluation of the Relative Frequency of Epstein–Barr Virus Infection in Patients with Recurrent Breast Cancer Compared with Patients with Nonrecurrent Breast Cancer

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Abstract

Background: The roles of Epstein–Barr virus (EBV) in breast cancer and breast lymphoma by transfecting EBV DNA have been indicated in different studies, but few investigations have been conducted on its roles in recurrence of breast cancer. Here, we aimed to evaluate the roles of EBV in recurrent breast cancer tissue.

Materials and Methods: This is a cross-sectional retrospective study that was performed in 2020–2021 in Isfahan on patients with breast cancer. The study population consisted of 30 tissue samples from recurrent breast cancer and 30 samples from nonrecurrent breast cancer. We collected demographic data of patients including age using a checklist. Other collected data were type of cancer, stages of cancer, tumor size in greatest dimension, lymph node involvements, and presence of metastasis. Furthermore, we evaluated all of the pathology samples from both groups for the presence of DNA of EBV and compared the data of both groups.

Results: The DNA of EBV was positive in 8 patients of the relapsed group (26.6%) and 7 patients in the nonrelapsed patients (23.3%). There was no significant difference between two groups regarding positive DNA of EBV ($P = 0.39$). There were no significant differences between two groups of positive DNA of EBV with and without recurrent breast cancer regarding type of cancer ($P = 0.63$), stage of cancer ($P = 0.19$), tumor size in greatest dimension ($P = 0.31$), mean lymph node involvement ($P = 0.27$), number of lymph node involvement ($P = 0.43$), and metastasis ($P = 0.69$).

Conclusion: EBV might have no significant role in recurrence of breast cancer.

Keywords: Breast neoplasms, neoplasms, recurrence

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Introduction

Breast cancer is the most common leading cause of death due to cancers in women with higher prevalence in 20–59 years of age.[1,2] Epidemiologic studies have reported that breast cancer is the second cause of death in the United States.[3,4] Although the prevalence of breast cancer is lower in Asian countries than in Western population, the prevalence is increasing.[5] Recent studies in Iran have reported that the prevalence of this cancer among Iranian women has reached 22% and unfortunately the age of onset of this disease in Iranian women is 10–15 years less than the age of onset in Western countries.[6-8]

Thus, given the high prevalence of breast cancer and its incidence and mortality worldwide, it seems that preventive strategies are the most important controlling methods. The screening methods include self-examinations, physical

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examinations, mammography, breast ultrasound, and magnetic resonance imaging.[5,9,10]

The cause of breast cancer is not fully understood, however, it is believed that genetic background and hormonal effects play an important role in its development.[11,12] According to the International Agency for Research on Cancer, 18%–20% of cancers might be related to infections. Exposure to a common virus such as Epstein–Barr virus (EBV), mouse breast tumor virus, and human papillomavirus has also been suggested as a risk factor for breast cancer.[13,14] Studies since 1995 have reported that EBV is involved in causing breast cancer.[15]

EBV infects about 90% of the world's population and usually carries a long-term, asymptomatic infection. EBV has also been reported with specific malignancies such as Burkitt's lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma.[16,17] The results of various studies showed that a logical link can be made between breast cancer and viral infection, the most important of which are the presence of EBV in breast tissue.[18,19]

The possibility that invasive ductal and lobular breast cancer may be associated with EBV was raised by Labreque *et al.* and led to further studies.[20-22] It has also been assumed that EBV could have pivotal roles in the recurrence of breast cancer in some patients. However, there are still much to discover in this regard.

Therefore, considering that the role of EBV on breast cancer recurrence after tumor surgery in our region has not been investigated, in this study, we aimed to investigate the relative prevalence of EBV infection in patients with recurrent breast cancer.

Materials and Methods

This is a cross-sectional retrospective study that was performed in 2020–2021 in Omid Hospital affiliated to Isfahan University of Medical Sciences. The current study was conducted on patients with breast cancer and a history of breast surgical operation. The study protocol was approved by the Research Committee of Isfahan University of Medical Sciences, and the Ethics Committee has confirmed it (Ethics code: IR.MUI.MED.REC.1399.479).

The inclusion criteria were female patients, diagnosis of breast cancer of any type by expert oncologists, history of breast surgical operation due to cancer, recurrent breast cancer, and signing the written informed consent to participate in this study. We should note that we considered recurrent cancer as recurrence in the same breast during 6 months to 1 year after previous surgery. The exclusion criterion was patient's will to exit the study.

Based on formula for estimating the sample size and 95% reliability equal to 1.96 and 0.80 for the test power, and the effects size of 0.62S, we considered 30 patients as the study population and 30 patients as controls. Patients were recruited randomly based on the mentioned criteria. Patients in the control group were recruited randomly from patients with breast cancer that underwent surgical operation but had no recurrence after 1 year after the operations.

We collected demographic data of patients including age using a checklist. Other collected data were type of cancer, stages of cancer, tumor size in greatest dimension, lymph node involvements, and presence of metastasis. Furthermore, we evaluated all of the pathology samples from both groups for the presence of EBV DNA and compared the data of both groups.

The DNA primers that were used were the following: for Epstein–Barr nuclear antigen1 gene:
F: 5′-GGATGCCTGGACACAAGAG-3′
R: 5′-TGACAAAATGGTGGGTGCTG-3′
For B-actin gene:
F: 5′-CATGTACGTTGCTATCCAGGC-3′
R: 5′-CTCCTTAATGTCACGCACGAT-3′

All tests were performed using SinaPure DNA formalin-fixed, paraffin-embedded tissue, EX6041.

The EBV DNA extraction was conducted as follows:

After selecting the samples, using a microtome machine, 6–10 sections were prepared from each block with 6-micron thickness and were poured into 1.5-ml microtubes without RNase/DNase. After deparaffinization of the samples with xylene (MERK, Germany) and absolute ethanol, genomic DNA was extracted using the salting-out method.

The extraction method was that 500 µl of lubricating buffer and 20–40 µl of proteinase k were added to each sample. The samples were then vortexed and spinned and placed in a water bath at 55°C overnight (if the tissue was not fully digested after 24 h, proteinase k was added again and incubated overnight at 55°C). Then, 200 µl of 5 M NaCl was added to each sample, and after centrifugation at 14000 rpm for 5 min, the supernatant was transferred to a new tube.

Sediment containing protein and salt was discarded. To the supernatant transferred to another vial, the same volume of cold isopropanol was added and was mixed thoroughly by stirring. To increase work efficiency, the microtubes were placed in a freezer at −20°C for 1 h. They were then centrifuged at 14,000 rpm for 20 min, and the supernatant was discarded. The precipitate was washed with 70% ethanol and then centrifuged at 14,000 rpm for 5 min. This step was repeated twice until the DNA was completely washed. Finally, after the precipitate was completely dry at room temperature, it was dissolved in an appropriate amount of water (to dissolve DNA in water, the extracted DNA was placed in a water bath at 55°C for 30 min). Samples were stored in a-20°C freezer until polymerase chain reaction (PCR) was done.

For evaluation of purity and quality of extracted DNA, we used Nanodrop apparatus. The optical density ratios of 260 nm to 280 nm were measured. Samples with a ratio of about 1.7–2 were used for PCR. Suitable samples for PCR were determined by amplification of beta-actin gene with a specific primer of that gene. To perform the reaction, a 25-µl mixture was

prepared for each sample according to the agenda provided for Master Mix PCR by Sinagen Company. First, they were heated at 94°C for 5 min and then for 30 cycles under PCR, including degreasing at 95°C for 1 min, connecting primers at 61°C for 50 s, and elongation at 72°C for 45 s. Finally, they were left at 72°C for 10 min to ensure that the product was fully propagated. PCR products were electrophoresed on 1.5% agarose gel to show a band of 161 bp.

Each sample that was positive for the beta-actin gene was retested for PCR to detect the EBV virus. At this stage, because the potential virus must multiply in the samples, the EBV primer was necessary. PCR samples were poured into a 0.2-ml microtubule, 10 µl PCR Master Mix (Ampliqon Company), and 0.5 µl from each reciprocating primer and 20 ng of template DNA. The final volume of the reaction was then reached to 20 microliters with deionized water. The temperature program for PCR of the EBV gene included the start-up phase at 94°C for 5 min, and 40 cycles including the step of inoculation of two DNA strands at 94°C for 45 s, binding of primers at 54°C for 45 s, and elongation at 72°C for 45 s and finally again at 72°C for 10 min to ensure that the product is completely elongated. To confirm the PCR products, 1.5% agarose gel was used for electrophoresis to show a band of 497 bp. Positive DNA control (virus genome) and negative control (water) were used at all stages to detect possible infections [Figure 1].

The obtained data were entered into the Statistical Package for Social Sciences (SPSS) (version 24, SPSS Inc., Chicago, IL, USA). Quantitative data were reported as mean ± standard deviation and qualitative data as frequency distribution (percentage). Independent *t*-test and Chi-square test were used to analyze the data $P < 0.05$ was considered as significance threshold.

Results

In the current study, we selected 30 samples from patients with recurrent breast cancer and 30 samples from patients without recurrence and evaluated the presence of EBV DNA in the selected samples.

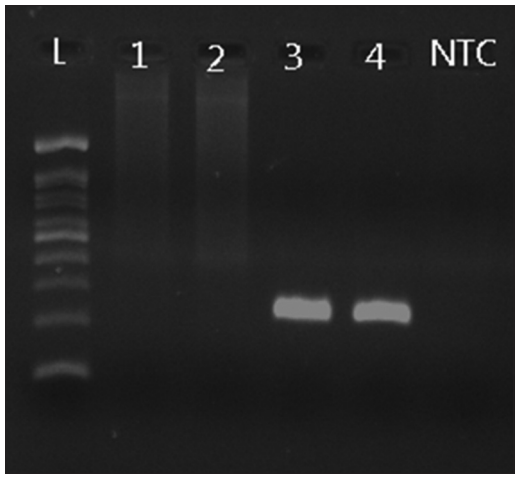

Figure 1: Gel bands regarding the DNAs. 1 and 2: Negative samples, 3: Positive samples, 4: Positive control

Based on the data, the EBV DNA was positive in 8 patients of the relapsed group (26.6%) and 7 patients in the nonrelapsed patients (23.3%). There was no significant difference between two groups regarding positive EBV DNA ($P = 0.39$).

In the next step, we compared different patient's data between the patients with positive EBV DNA. Based on these data, there were no significant differences between two groups regarding mean age of patients and age categories ($P = 0.28$ and $P = 0.36$, respectively). These data are presented in Table 1.

We also compared different cancer characteristics between two groups of positive EBV DNA with and without recurrent breast cancer. These data indicated that there were no significant differences between two groups regarding type of cancer ($P = 0.63$), stage of cancer ($P = 0.19$), tumor size in greatest dimension ($P = 0.31$), mean lymph node involvement ($P = 0.27$), number of lymph node involvement ($P = 0.43$), and metastasis ($P = 0.69$). Based on the presented data, the most common type of cancer in these patients was ductal invasive carcinoma (84.4%), the most common cancer stage was 3 (43.8%), most patients had 1–3 lymph node involvements (34.5%), and they had equal frequencies of metastasis. These data are indicated in Table 2.

Discussion

The importance of EBV in cancer recurrence has been mentioned earlier. In the present study, we evaluated 60 patients with breast cancer and found that there were no significant differences between two groups of relapsed and nonrelapsed breast cancers in terms of positive EBV DNA. Furthermore, we observed no significant differences between cases with positive EBV DNA and other cases regarding type of cancer, stage of cancer, tumor size, lymph node involvement, number of lymph node involvement, and metastasis.

These data cast doubt on the roles of EBV in recurrent breast cancer. In this regard, some studies have been conducted that evaluated the roles of EBV in breast cancer. Most of the previous studies have assessed the roles of EBV in formation and aggression of breast cancer and less attention has been given to the issue of recurrence. The results of these studies were somehow controversial, and no definite relationship between EBV and recurrence was reported. The important point of this study was that we evaluated the positivity of EBV DNA in two groups of breast cancer tissues with and without recurrence and observed no significant differences. In 2017, a study was conducted by Naushad *et al.* in Pakistan. By 250 breast cancer tissues resulted from biopsies, they reported that EBV DNA was observed in 24.4% of samples. However, this issue had no significant relationships with recurrence in patients.[23-25] Another study by Mofrad *et al.* in 2020 evaluated the prevalence of EBV in Iranian breast carcinoma patients. Based on this study, among 59 carcinoma samples, 6.7% had

Table 1: Comparison of the age and age groups in patients

Variable	Positive EBV DNA with relapse (*n*=8), *n* (%)	Positive EBV DNA without relapse (*n*=7), *n* (%)	Total (*n*=15), *n* (%)	*P*
Age (years), mean±SD	51.26±11.27	52.09±10.82	15 (100)	0.28[a]
Age category (years)				
30-40	0	1 (14.3)	1 (6.7)	0.36[b]
41-50	3 (37.5)	3 (42.9)	6 (40)	
51-60	4 (50)	2 (28.6)	6 (40)	
61-70	1 (12.5)	1 (14.2)	2 (13.3)	

[a]Independent samples *t*-test, [b]Chi-square test. SD: Standard deviation, EBV: Epstein-Barr virus

Table 2: Comparison of different cancer characteristics between two groups

Variable	Positive EBV DNA with relapse (*n*=8), *n* (%)	Positive EBV DNA without relapse (*n*=7), *n* (%)	Total (*n*=15), *n* (%)	*P*
Type of cancer				
Ductal invasive carcinoma	6 (85)	6 (85.7)	12 (80)	0.63[a]
Lobular carcinoma	2 (15)	1 (14.3)	3 (20)	
Stage of cancer				
1	1 (12.5)	1 (14.3)	2 (13.3)	0.31[a]
2	2 (15)	1 (14.3)	3 (20)	
3	2 (15)	4 (57.1)	6 (40)	
4	3 (37.5)	1 (14.3)	4 (25)	
Tumor size in greatest dimension (mm)				
0-20	1 (12.5)	2 (28.5)	3 (20)	0.31[a]
21-50	7 (87.5)	5 (71.5)	12 (80)	
>50	0	0	0	
Lymph node involvement (mean±SD)	2.09±0.69	1.82±0.58	1.93±0.82	0.27[b]
Lymph node involvement (node)				
0	3 (37.5)	2 (28.5)	5 (33.4)	0.43[a]
1-3	2 (15)	3 (42.9)	5 (33.4)	
4-5	2 (15)	1 (14.3)	3 (20)	
<6	1 (12.5)	1 (14.3)	2 (13.2)	
Metastasis				
Yes	4 (50)	3 (42.9)	7 (46.6)	0.69[b]
No	4 (50)	4 (57.1)	8 (53.4)	

[a]Chi-square test, [b]Independent samples *t*-test. SD: Standard deviation

positive EBV DNA.[26] All these data were in line with the results of our study, but the important point is that we assessed the roles of EBV in recurrent breast cancer and observed no significant relationships.

Furthermore, some previous studies have shown controversial findings about the roles of EBV in breast cancer. For instance, Abdallah *et al.* revealed that EBV has an important role in development of breast cancer. Furthermore, they showed that epigenetic assays have high value in interrogating breast cancer tumorigenesis, and pinpointing specific developmental and viral pathway dysregulation. They also suggested that these could serve as potential biomarkers or targets for therapeutic interventions.[27-29] On the other hand, Saeedi *et al.* performed a study in 2018 in Iran. In this study, they reported that EBV was found in 5.12% of breast cancer tissue but reported that there were no significant relationships between breast cancer and EBV.[30] These differences could be justified based on the regional differences. In 2021, a study was performed by Sinclair *et al.* in the United Kingdom. This study assessed the relationships between EBV and breast cancer and showed that the evidence for the presence of EBV in breast cancer biopsies is concentrated in specific geographic regions but is currently insufficient to provide a causal link.[31,32]

On the other hand, a study was performed by Farahmand *et al.* in 2019 that evaluated the roles of EBV in breast cancer. Based on this systematic review, there was a strong statistical relationship between EBV infection and risk of breast cancer.[33,34] Based on the results of Mazouni *et al.* in 2015, EBV positivity was found to exert no effect on survival, despite its association with aggressive breast cancer phenotypes.[35,36] Our results were not in line with these data because we detected EBV DNA in 26.6% of cases with breast cancer. However, we observed no significant relationships between presence of EBV and recurrent breast cancer. We believe that these differences could be due to variations in the study population and regional differences.

Here, we had a retrospective evaluation of patient's documents. The limitations of this study were that this study could have unknown potential confounders, we used the data that were originally collected for these purposes, not all the relevant information, and we had also inferior level of evidence compared with prospective studies. We also had restricted study population compared to some former studies and, therefore, suggest that more studies on larger populations should be performed.

Conclusion

Based on our data, EBV might have no significant role in recurrence of breast cancer. Our data, however, indicated the importance of EBV in development of breast cancer and were in line with previous data.

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Conflicts of interest

There are no conflicts of interest.

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