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A comparative study of Epstein-Barr virus (EBV) in malignant tumors and fibrocystic lesions of the breast

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ABSTRACT

Breast cancer is the most common non-skin malignancy and the second leading cause of death due to cancer in women (after lung cancer). Several factors involved in this disease have been studied. EBV has been proposed as a possible cause of breast cancer in humans. Information related to the possible association of EBV with breast cancer is contradictory and inconclusive. Therefore, we started to examine the role of this virus in breast cancer. The study was conducted on paraffin-embedded samples of patients with breast cancer (case or experimental group) and fibrocystic lesions (control group) available in the archives of pathology laboratory of Shahid Beheshti Hospital of Kashan related to 2012-2013. Samples DNA was extracted and infection with EBV virus was analyzed using PCR method. SPSS-21 was used for data analysis. The frequencies were compared with Chi-square test and the means with t-test. In this study, out of 40 patients with cancer, 14 patients (35%), and out of 40 patients with fibrocystic lesion, 25 patients (62.5 percent) were positive regarding EBV virus (p=0.02). In the samples of cancer, no significant differences were obtained between the two groups of positive and negative EBV in terms of average age (p=0.38), average menarche age (p=0.16), average age of menopause (p=0.18), and the average age the first birth (p=0.97). In addition, no statistically significant differences were observed between positive and negative groups in terms of tumor size. The results of this study showed that EBV is significantly more in fibrocystic lesions than in breast cancer. Thus, no etiologic relationship was found between EBV infection and Invasive Ductal Carcinoma (IDC) of Breast in this study, and more research is needed to clarify the role of this virus in causing breast cancer.

Key words: breast cancer, Epstein-Barr virus, fibrocystic lesion

INTRODUCTION

Breast cancer is the most common malignancy [excluding skin cancer] among women worldwide. In 2012, one point sixty seven million new cases of this cancer were reported, and 25% of all reported cancer cases were among

women. This form of cancer in developing countries is a little more than what it is in developed countries [about 883000 cases against 794000] [1].

Breast cancer is the most common cause of death due to cancer in women in developing countries and the second in developed countries, and accounts for 15.4% and 14.3% of the all death causes among the women of these countries and every year 324,000 people in developing countries and 198000 people in developed countries die due to this [1]. The risk factors of breast cancer in women include increasing age, early menarche, late menopause, nulliparity or lactation, high levels of estrogen, race, family history of breast cancer, lifestyle, obesity, alcohol consumption, various genes [including BRCA1 and BRCA2] and viruses [3,2].

The studies carried out during the last two decades have raised the role of viruses in causing breast cancer. One of the most important viruses discussed in causing breast cancer is EBV. This virus is a member of herpesviridae family [4] and is a carcinogenic virus and it has a role in cancers such as Burkitt's lymphoma [5], Hodgkin's lymphoma [6], nasopharyngeal cancer [7], lymphoproliferative disorders [8], and stomach cancer [9]. There are different methods to detect EBV virus, such as Immunohistochemistry [IHC] [10], in situ hybridization [ISH] [11], Southern Blot Hybridization [12], and PCR [13], among which PCR is the most sensitive and specific method of isolation of EBV genetic material [12].

Some studies suggested a link between breast cancer and EBV:
Yahiya et al. [2014] investigated the role of EBV in breast cancer incidence in Sudan. In this study, 92 breast-cancer biopsy samples and 50 samples of adjacent normal tissue were collected from people who had undergone surgery and had not received any anticancer drug. According to the results obtained from this study, using LMP-1 and EBNA-4 primers, EBV genome was identified in, respectively, 11 and 53.3 percent of cancer cases. In the control group samples, using LMP-1 primer, EBV genome was detected in 24% of patients, and this while using EBNA-4 primer, all control group samples were negative. There was statistically a significant difference between the two groups of cancer and control samples using primers LMP-1 and EBNA-4 [p=0.001] [14].

In the study by Tahmasebifard [2013] conducted in Iran, paraffin embedded samples of 65 patients with breast cancer and 53 samples of breast tissue from women with fibrocystic disease were studied as the control group. According to the findings of this study, EBV genome was revealed in 35.38% of the cancer group samples and in 20.75% of fibrocystic group [control group]. The results indicated a statistically significant relationship between EBV and breast cancer [15].

In the study by Eqbali et al. [2012] in Iran on 24 patients with breast cancer and 24 patients with fibroadenomas referring to Toos and Firoozgar hospitals in Tehran during 2011-2012, PCR technique was used and the prevalence of EBV in malignant samples was higher than in benign tumors. Sixteen point six percent of the patients with breast cancer and 4.1% of patients with fibroadenomas had been infected with EBV, suggesting a higher prevalence of EBV infection in cancer samples [16].

A study was conducted by Khabaz [2012] in Jordan on 92 paraffin-embedded breast cancer samples and 49 control paraffin embedded blocks [including fibrocystic, fibroadenomas, sclerosing adenosis, tubular adenomas, and intraductal papillomas] in the Department of Pathology of the University of Jordan using PCR and immunohistochemistry methods on Epstein-Barr nuclear antigen 1 [EBNA-1]. In the study, 24 cases of the 92 cases of breast cancer were infected with EBV, while three cases of the 49 cases of control samples were positive regarding EBV [p=0.008]. In immunohistochemistry method, in 24 cases of 92 cases of breast cancer, Epstein-Barr nuclear antigen 1 [EBNA-1] was positive. The results of this study show the association between EBV infection and the development of breast cancer, but no relationship was observed between EBV-induced cancers and age, grade, and size of tumor [17].

Aguayo et al. (2011) studied the relationship between EBV infection and breast cancer. In this study, DNA of 55 paraffin-embedded breast cancer samples was extracted by PCR. According to the results obtained from this study, in 6.5% of samples, EBV genome was detected. According to the results obtained from this study, the researchers stated that EBV plays a direct role in causing breast cancer, but no statistically significant relationship was observed between EBV with patient age (p=1), lymph node involvement (p=1), tumor size (p=0.495), histology (p=0.130), and differentiation grade (p=0.255) (18).

In the study by Mohammadizadeh et al. (2010) on 80 paraffin-embedded breast cancer samples in Al-Zahra hospital, Esfahan, latent membrane protein 1 (LMP-1) infection was assessed by immunohistochemistry. LMP-1 expression was seen in six cases (7.5 percent) of breast cancer, while the tissue adjacent to the tumor did not show the expression of this protein in any case. In this study, the association between EBV infection and invasive breast carcinoma was shown (p=0.03). No relationship was observed between the expression of LMP-1 and age, tumor size, tumor grade, and lymph node involvement (19).

In the study by Bonnet et al. (1999) conducted in France on 100 paraffin-embedded breast cancer samples (PEBCS) and 30 samples of normal tissue adjacent to cancer at the Institute of Pathology of Gustave Roussy, EBV genume was expressed in 51% of tumor tissues by PCR, while in 90% of normal adjacent tissue, tumor was not observed ($p<0.001$). The results of this study indicate the association of EBV with breast cancer virus and its role as a cofactor in the development of breast cancer (20).

In some studies, EBV genetic material was not found in samples of breast cancer:
In a study by Torabizadeh et al. (2014) in Iran that was conducted on 79 PEBCS and 51 fibroadenomas samples by PCR in Emam Khomeini Hospital of Sari, EBV virus became positive in four cases of invasive ductal cancer (7.3 percent) and in one case (2.4 percent) of fibroadenoma samples ($p>0.05$). In this study, no significant association was observed between EBV infection with breast cancer and the patient's age and in tumor grade with EBV infection (21).

In the study conducted by Fadavi et al. (2013) on paraffin-embedded samples of 18 breast cancer patients referring to Tehran Pars Hospital by PCR, EBV genome was not detected in any of the samples, so they stated that probably EBV has no significant role in causing breast cancer (22).

Kadivar et al (2011) studied 100 samples of breast cancer and 42 biopsy samples of fibrocystic lesions (control group) in terms of EBV virus. In this study, EBV virus was not detected in any cancer and biopsy samples of the control groups. In conclusion, it was stated that the virus has no significant role in the incidence of breast cancer in Iranian women (23).

Deshpande et al. (2002) conducted a study in America on 33 women over 50 years and 10 women under 50 with breast cancer. The mass size was from 0.8 to 8.5 cm, in most of which (23 cases), it was less than two cm. Thirty-six patients had infiltrating ductal carcinoma, two had infiltrative lobular carcinoma, and five cases had mixed ductal-lobular morphology. In this study, hybridization and PCR methods were used to detect EBV genome, and EBV genome was not found in any of the 43 samples studied (24).

Herman et al. (2002) conducted a study in Germany on 59 samples of invasive breast carcinoma obtained from the records of Institute of Pathology of the University of Friedrich. In this study, PCR was used to detect EBV DNA, in situ hybridization was used for EBV DNA, in situ hybridization for EBERs, and immunohistochemistry was used for the demonstration of the EBNA1, where in situ hybridization for EBERs and viral DNA and immunohistochemistry for EBNA1 were negative in all cases. However, using PCR, in four out of 59 cases (6.8 percent) were reported to be positive that was not statistically significant (25).

MATERIALS AND METHODS

By referring to the archives of Shahid Beheshti hospital pathology lab in Kashan, the blocks made from breast lumpectomy and mastectomy in 2012 and 2013 related to the patients of different age groups, whose pathological diagnoses were fibrocystic lesions and invasive ductal carcinoma were selected. Moreover, the demographic information of the patients including age, menarche age, menopause age, first birth age, duration of hormone replacement, family history, type of lesion, and tumor size were extracted and recorded.
In short, implementation phases of this project were as follows:

Preparation of tissue sections out of paraffin-embedded blocks
After selecting the best and most appropriate paraffin-embedded blocks available at the archive of the pathology lab, 10 slices with a thickness of 3.5 microns was prepared from cross-sectional area of each block under sterile conditions by microtome, poured into micro tubes, were labeled, and were kept in the refrigerator until deparaffinization step.

Deparaffinization:
1. In each tube containing tissue sections, 1000 microliter of Xylen solution was poured and was gently inverted, and then they were vortexed three times and each time four seconds.
2. The samples were placed in Dry block for 10-15 minutes at a temperature of 37 ° C.
3. The samples were centrifuged at 25 ° C for 10 minutes at 14,000 round per minute ($_{rpm}$) and 800-900 ml of the supernatant was removed by pipetting.
4. 1000 microliter of Xylen was added to each tube. Then they were vertex three times and each time four seconds at a temperature of 30 ° C for 10 minutes with 14000-rpm and thorough centrifuge, supernatant was removed completely.
5. 1000 ml of 100% ethanol was added to each tube and after quick inversion, it was vertex three times and each time four seconds.
6. The tubes were placed at room temperature for 15 minutes, and then were centrifuged for 10 minutes at 25 ° with 14000 rpm, and 800-900 ml of supernatant was discarded by pipette.
7. 100% ethanol was added in the same way for the second time and was left for 30 minutes at room temperature. After centrifugation with the same conditions as above, the supernatant was depleted.
8. 1000 ml of 70% ethanol was added to each tube and was gently inverted, then three times and each time for seconds was vortex, and the tubes were placed for 30 minutes at room temperature. Then they were centrifuged for 10 minutes at a temperature of 22 ° C with 14000 rpm and the supernatant was depleted.
9. For drying the contents of the tube, they were placed at a temperature of 37 ° C for 15 minutes in a dry block.

Tissue Lysis:
After deparaffinization phase, 100 ml of lysis solution tissue (prelysis Buffer) was added to the deparaffinized tissue and 10 ml of rhybotinasis made by Sinaclon Company Iran was added and incubated for 1 to 3 hours at 55 ° C.

DNA Extraction:
DNA extraction stages according to the instructions of the used kit, Amplisens RIBO-prep nucleic acid extraction kit, made in Russia are as follows:
1. As required according to the number of samples (80), we took and labeled 1.5 ml tubes plus two tubes (negative control and positive control of extraction process).
2. We added 10 ml internal control and then 300 ml of lysis solution (solution for lysis) to the patients' tubes.
3. We added 100 ml of the lysate tissue in colloidal form to the tubes of the patients, 100 ml negative control to negative control tube, 90 microliters of negative control, and 10 microliters of positive control to positive control tubes.
4. We sealed the pipes, well mixed their contents, and incubated for 5 min at 65 ° C.
5. Precipitation stage: we added 400 ml of solution for precipitation to the sample and centrifuged it for 5 minutes at 13000 rpm. The supernatant was removed and the next steps were done on the precipitation.
6. The first washing phase: 500 ml of washing solution 3 was added to tubes the tubes were inverted 2 to 4 times, and centrifuged for 2 minutes at 13000 rpm. The supernatant was removed and the next steps on the precipitation.
7. The second washing phase: 200 ml of washing solution 4 was added to tubes the tubes were inverted 2 to 4 times, and centrifuged for 2 minutes at 13000 rpm. The supernatant was removed and the next steps on the precipitation.
200 ml of washing solution 4 (Washing Solution 4) to
Join pipes and tubes 2 to 4 times the upside were centrifuged for 2 minutes in rp-13000. The supernatant was removed and the next steps on the scale.
8. Precipitation of tubes was incubated for five minutes at 65 ° C and changes to semi-arid state.
9. Fifty ml of buffer (RNA-buffer) RNA was added to the tubes precipitation, well mixed, and incubated for 5 min at 65 ° C.
10. We centrifuged tubes for 1 min at 13000 rpm, transferred the supernatant containing DNA into a new tube, labeled, and kept in the refrigerator until PCR.

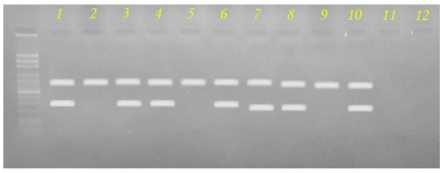
PCR:
PCR or polymerase chain reaction is one of the most widely used techniques in molecular biology, which allows the amplification of one or a number of copies of DNA to thousands and millions of times.

After extracting the DNA, in the next step, PCR method was conducted using kits EBV-Eph PCR kit variant 100R manufactured by Amplisens Co., Russia according to the instructions in the kit. The final volume of the tube in PCR reaction is 25 micro-liters, and 10 ml is related to DNA.

1. As required according to the number of samples (80), we took PCR tubes in the kit PCR-mix-1-R EBV ready-to-use single-dose test tubes (under wax) plus two tubes (negative control and positive control of extraction process).
2. We added 10 ml of blue solution PCR-mix-2 to the wax layer of all tubes.
3. We added a drop (25 microliter) of mineral oil to all tubes.
4. We added 10 ml of the extracted DNA from tissue samples to the patients' tube.
5. We added 10 microliter of DNA of buffer to the negative control PCR tube and added 10 ml of positive control EBV / hDNA to PCR positive control tube.
6. We placed the tubes in the thermocycler device BIO-RAD Made in America.
PCR was done by thermocycler device.

Electrophoresis:
Electrophoretic of separation of charged molecules is based on their electrical charge. This method is used for separating ionized substances including amino acids, nucleic acids, organic acids, and small anions and cations. To carry out electrophoresis, we placed ten lambda of the PCR on agarose gel wells, and then transferred the gel into the electrophoresis tank so that the wells were at the negative pole of the tanks. Then we connected the tank to the current-voltage 100V, and after 30 minutes, we removed the gel and photographed it by the imaging machine Gel Doc.

Reading and interpreting gel and bands
Photos taken from agarose gel are observed and examined as follows.
1. In the event that both bands 290 bp (related to DNA virus) and band 723 bp (related to internal control DNA) are seen on agarose gel, the sample is considered positive.
2. In the case of observing 723 bp and not 290 bp, the sample is considered negative.
3. Positive control sample has both bands 723 bp and 290 bp. PCR negative control sample and the extraction process lack both bands.
4. In the samples lacking both 723 bp and 290 bp bands in agarose gel, DNA extraction step test was repeated.
Samples of PCR on electrophoresis gel are shown in the following figure. Sample (10) is related to the positive control PCR, 11 is PCR negative control, and 12 is the negative control of extraction process. The samples 1, 3, 6, 7, and 8 are considered positive and 2, 5 and 9 are considered negative.



Samples of PCR on electrophoresis gel

Data analysis and statistical analysis:
SPSS-21 was used for data analysis. Mean, standard deviation, median, range, frequency, and percentages were used for descriptions of the data and charts and tables were plotted. Quantitative variables were expressed as mean and standard deviation and qualitative variables as frequency and percentage. Frequencies were compared with chi-square test and t-test. To express the estimation accuracy, 95% confidence interval was used.

RESULTS

Out of all patients with cancer in this study (n= 40), 14 patients (35%) were positive for EBV, and out of those with fibrocystic lesions (n=40), 25 (62.2%) were positive for the virus. There was statistically a significant difference between the two groups, in terms of having EBV (p=0.02), so that in the group with cancer, EBV genome was less. (Table 1)

Table 1: Frequency of EBV-positive individuals in cancer and fibrocystic lesions groups

	EBV-positive group		EBV-negative group		Total		*P*-value
	Frequency	Percent	Frequency	Percent	Frequency	Percent	
Cancer	14	35	26	65	40	100	0.02
Fibrocystic lesions	25	62.5	15	37.5	40	100	

The average age of patients with cancer who were examined was 49.05 with a standard deviation of 9.57 years. The age range of these patients was 29-67 years. The average age of menarche of the patients was 13.27±1.15 years with age range of 9-15 years. The average age of menopause in the patients was 46.18±3.51 years with a minimum of 40 and maximum of 56 years. The mean age at first birth was 19.51±3.78 years with a minimum of 14 and maximum of 30 years.

The average age of EBV positive patients with cancer (positive group) was 50.85±9.79, with age range of 29-65. Age of menarche in these patients was 12.92±0.73 with the range of 12-14 years. Moreover, the age of menopause in EBV positive patients was 45.5±3.14 years. The menopause age range in these patients was 41-51 years. Average age at the first delivery in these patients was 19.50 with a standard deviation of 3.99 (Table 2).

The average age of EBV negative patients with cancer (negative group) was 48.07, with SD of 9.50, and range of 30-67. Average age of menarche in these patients was 13.46±1.30 with the range of 9-15 years. Moreover, the age of menopause in EBV negative patients was 46.44 with SD of 3.71, and range 40-56. Average age at the first delivery in these patients was 19.52 with range of 14-30 (Table 2).

According to the results obtained from this study (presented in Table 2), there were no significant differences between positive or negative groups in terms of mean age (p=0.38), the average age of menarche (p=0.16), mean age of menopause (p=0.18), and the mean age at first birth (p=0.97).

Average size of tumor in EBV-positive patients was 5.21±2.70 with a range of 2-11 cm, and in EBV-negative group, it was 3.83±1.91 with a range of 1.5-9 cm. In fact, it can be argued that there is no statistically significant difference between the two positive and negative groups regarding tumor size (p=0.06) (Table 2).

Table 2: Comparison of information of patients with cancer into two EBV positive and negative groups

Variable		Mean	*P* value
Age (years)	EBV positive group	50.85	0.38
	EBV negative group	48.07	
Menarche age (years)	EBV positive group	12.92	0.16
	EBV negative group	13.46	
Menopausal age (years	EBV positive group	45.54	0.18
	EBV negative group	46.44	
Age at first birth (years)	EBV positive group	19.50	0.97
	EBV negative group	19.52	
Age at first birth (years)	EBV positive group	5.21	0.06
	EBV negative group	3.83	

In EBV-positive group (n=14), the frequency of tumor grade in 10 patients (71.42 percent) was two, in four patients (28.57 percent), it was three, and in EBV-negative group, 25 patients had grade two malignancy that had the most frequency (96.15 percent) among the patients (Table 3).

Of the all patients with cancer in this study (n=40), four patients had a family history of breast cancer, of whom one was positive in terms of EBV and three were negative.

Table 3: Frequency of Tumor grade in EBV positive and negative patients with cancer

		Frequency	Percent
EBV positive group	Grade 2	10	71.42
	Grade 3	4	28.57
	Total	14	100
EBV negative group	Grade 2	25	96.15
	Grade 3	1	3.84
	Total	26	100

DISCUSSION AND CONCLUSION

Breast cancer is the most common malignancy (excluding skin cancer) among women worldwide, with about 25 percent of total cancers and the most common cause of deaths due to cancer in developing countries (1). Breast cancer is a multifactorial disease. During the last two decades, the role of viruses such as EBV and HPV has been

proposed in breast cancer, but has not been established yet (4). Proving the etiologic role of special viruses in causing breast cancer may prove useful in early detection and prevention of breast cancer in high risk populations infected in the future.

According to the results obtained from the present study, out of 40 patients with cancer, 14 patients (35%), and out of 40 patients with fibrocystic lesion, 25 patients (62.5 percent) were positive regarding EBV. As can be seen, frequency of EBV is significantly (p=0.02) higher in patients with fibrocystic lesions than patients with breast cancer. Thus, no etiologic relationship was found between EBV infection and breast cancer in this study, but as in this study, the load of the virus was not determined, there might be virus in higher loads in cancerous lesions compared to fibrocystic lesions, and thus it might implement its oncogenic effects at higher loads.

In the present study, no significant differences were obtained between the two EBV positive and negative cancer groups in terms of mean age (p=0.38), the average age of menarche (p=0.16), mean age of menopause (p=0.18), and the average age at the first birth (p=0.97). Moreover, no significant differences were observed between EBV negative and positive cancer groups in terms of and tumor size (p=0.06).

Different prevalence of association of EBV with breast cancer in studies on depends on several factors including sample size, sample type (using blocks of paraffin or fresh tissue), and epidemiological factors including differences at the time of acquiring primary infection of EBV. Moreover, it depends on different prevalence of EBV in different parts of the world, and the type of methodology used in the detection of viruses (19). Moreover, since interleukin-10 (IL-10) and interferon gamma (INF-γ) play a vital role in the host response to infection with EBV, genetic variations, particularly in interferon gamma, may increase or moderate the companionship of EBV virus infection with breast cancer (21).

Most studies conducted have shown the existence of virus genome in cancer samples using PCR technique. Compared to PCR, techniques such as in situ hybridization and immunohistochemistry mostly lead to negative results that could be due to the fact that in situ hybridization and immunohistochemistry can only show the expression of active viral genome, while PCR make detection of latent form of the virus possible (25). In addition, the use of different primers in the PCR method to detect EBV genome in cancer and controls samples is of the reasons for differences in PCR results in different studies. Moreover, in the study by Yahiya et al., by using two different primers in cancer and control groups, different results were observed in both groups (14). Another factor in the detection of EBV virus in cancer samples is the hypothesis of the presence of EBV virus only in infiltrating lymphocytes in cancer samples. However, this hypothesis has not been confirmed in some studies (16).

Paradoxical results of EBV virus with breast cancer need more studies to achieve reliable results. Serological studies to determine the lifetime of exposure to EBV infection in new samples of breast cancer and simultaneous study of breast cancer markers in breast tissue of seropositive individuals, as well as quantitative PCR studies to determine the viral load in cancerous samples and healthy adjacent tissue could show a more clear vision of the role and association of EBV in breast cancer.

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