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Association between Epstein-Barr Virus (EBV) and Breast Cancer

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Abstract

Background: Breast cancer is the most common malignancy in females worldwide. Several etiological factors including environmental factors have been recognized for breast cancer. Epstein Barr virus as a viral etiological factor has been proposed. So far, several studies have investigated the relationship between development of breast cancer and Epstein Barr virus, but few have been done in Iran. The aim of this study was to determine whether there was an association between EBV infection and female breast cancer in Iran.

Materials and Methods: We analyzed paraffin embedded breast tissue specimens by polymerase chain reaction (PCR) including breast cancer specimens (as case group) and breast fibroadenoma specimens (as control group). PCR was performed to amplify specific sequences of EBV.

Results: From 130 cases of breast samples, 67 cases of breast cancer tissues and 41 cases of breast fibroadenoma tissues had adequate quality and quantity of DNA to detect EBV. PCR for EBV was positive in 4 invasive ductal carcinoma specimens (7.3%) and only one of the fibroadenoma specimens (2.4%). No significant association was found between EBV infection and invasive ductal carcinoma ($p > 0.05$). Also, patient's age and histological grade of IDC were not correlated with EBV infection ($p>0.05$).

Conclusion: We observed no etiologic association between EBV infection and invasive ductal carcinoma of female breast in our regions; however, further studies are required to elucidate this association.

Keywords: Breast cancer; Fibroadenoma; Epstein-Barr virus; Polymerase chain reaction

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Introduction

Breast cancer is the second reason of mortality in the world (1, 2) and the occurrence has increased by 2-fold over the past 30 years (2). The prevalence of breast cancer is 23% among all cancers in the world (3), and its mortality rate is about 16% (1), so it is the most common and fatal cancer in women (2,4). Risk factors of breast cancer are age, family history, menarche, delayed menopause, first pregnancy after 25 years of age, nulliparity, long-term consumption of exogenous estrogens, and obesity after menopause, and encountering ionizing ray (5). Epstein –Barr virus (EBV) has also been found to be as an etiological reason for breast cancer (5). This virus was first recognized by two researchers, Barr & Epstein in 1964 (6). It belongs to Herpesviridae family with subfamily of Gamma herpesviridae that is called Human herpes virus-4 (HHV-4) too. This virus had linear double strands DNA (7). The most important way of transferring EBV is through direct oral contact and saliva, while contamination through transfusion, transplantation and placenta is also possible (6, 8). Rate of involving with this virus is about 100% in developing countries (9, 10, 11). Persistent lifetime infection stems from involvement of memory B-cells which are the main source of virus and amount of infection is between 1 to 50 infected cells per 10^6 B cells in healthy seropositive people (11). This virus causes some diseases such as

infectious mononucleosis (IM), Burkett's lymphoma, nasopharyngeal carcinoma (NPC), oral hairy leukoplakia, multiple sclerosis (MS), dendritic follicular cells malignancy (due to CD 21), Hodgkin's lymphoma, Non-Hodgkin's lymphoma, and gastric cancer (9,12-18,19). Some researchers indicated that EBV could change epithelial cells, and move toward malignancy (20-22). It is proved that EBV infection has no effect on c-myc and Bcl-2 -anti-apoptotic molecules-overexpression (23). It is shown that it can suppress Bax molecule expression, which plays a role in cellular apoptosis; thus, that could halter apoptosis in gastric cancer (24). This virus causes B cells growth and proliferation in Burkitt's lymphoma which is mediated by stimulation of IL10 production (25). So far, the association between EBV infection and breast cancer has not been expressed, and there are many controversies in this regard. Some studies implied an association between EBV and type of breast cancer (26, 27). On the other hand, others found no evidence of EBV infection (28-34). To the best of our knowledge, there are only a few studies about EBV in breast cancer in the Middle Eastern countries and few are from Iran as well. Therefore, in this study, we intended to investigate the presence of EBV in breast cancer specimens using polymerase chain reaction (Nested-PCR).

Materials and Methods

In this retrospective case-control study, EBV genome of 79 paraffin embedded breast cancer tissue samples were used from pathology department of Bouali Sina and Imam Khomeini hospitals, Sari. The samples were 67 IDC cases (Invasive ductal carcinoma), 8 cases of ILC(Invasive lobular carcinoma) 2 cases of MC(medullary carcinoma) and 1 case of DCIS(Ductal carcinoma in situ), 1 case of IDC- ILC , and 51 cases of paraffin embedded tissue samples of fibroadenoma as controls for Nested-PCR. All samples belonged to female patients. Primary characteristics such as age, tumor type, and tumor grade were obtained from patient's records and two experienced pathologists reconfirmed breast cancer diagnosis. 5-7 micrometer cut sections were prepared from all paraffin blocks and frozen to -70 °C.

PCR procedure was briefly;

1. Deparaffinizing formalin-fixed breast cancer paraffin blocks
2. DNA extraction from breast cancer cell
3. Amplifying DNA
4. Agarose gel electrophoresis

1. Deparaffinizing stage;

At first all samples were placed in xylene for 10 minutes and again in new xylene solution for 10 minutes consequently. Then, they were put in ethanol 100%, 95%, and 70% for 5 minutes, respectively. Finally, they were placed in distilled water for 5 minutes (10).

2. DNA extraction (in Nested-PCR);

DNA was extracted by using extracting kit produced by Iran KIAGEN called purification kit. All DNA samples were stored in -20 ^{o}C in freezer until the time of PCR test. General primers sequences used in nested-PCR for diagnosis of EBV genome were outer primers such as EBVS1 (sense) d(CTACAACAAAA CTGGTGGACT) and EBVA1 (antisense) d(AGAC AGTGTGGCTAAGGGAGT) and inner primers such as EBVS2 (sense) d(TGCTCTCAAAACCTAGGCG CA) andEBVA2 (antisense) d(TGATTAGCTAAGG CATTCCCA).

Table 1. Comparison of case and control group for age and number of infected samples with EBV.

Variable	Breast cancer	Fibroadenoma	P-value
Age± SD (year)	48.05±12.5	34.2±9.7	<0.05
EBV-infected sample NO.	4(7.3%)	1(2.4%)	>0.05

Constituents of 50 microliter of primary PCR-mixture were as below;

1st round PCR	5µ
10xPCR buffer	2µ
50mM MgCl 2	1µ
Fast start DNA polymerase (5u.µl)	0.5µl
Primer S1 (10 µM)	5µl
Primer A1 (10µM)	5µl
PCR water	27µl
DNA Template	5µl

Distilled water for injection and EBV-infected cells was used as negative and positive controls, respectively.

2nd round PCR	
10xPCR buffer	5µl
50mM MgCl2	2µl
dNTps (10mM each)	1µl
Fast start DNA polymerase (5u.µl)	0.5µl
Primer S2 (10 µM)	5µl
PrimerA2 (10µM)	5µl
PCR water	27µl
1st round PCR product	5µl

3-Amplification in PCR process using external and internal primers was arranged in 35 cycles with three programs as follow;

1- One cycle consist of: 94 $^{\circ}$C for 1 min, 55 $^{\circ}$C for 1 min, and 72 $^{\circ}$C for 1 min. Then amplification was

followed for 33 cycles as; 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Finally PCR was down in one cycle as 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 7 min.

4- Agarose gel electrophoresis;
At first, agarose gel was covered by buffer (25 cc boric acid containing ethidium bromide in 500 cc distilled water), then, 10-15 µl of PCR solution and negative and positive controls were added to the gel. Electrophoresis was done for 15-20 minutes. Later on, the gel was taken out and the amplified bands were read by Gel Doc.

Interpretation
No band has been seen in negative control group. Two bands have been seen in positive control group. EBV-related bands (267 to 325 bp) and bands related to human-DNA (723 bp) were identified.
Statistical analysis: collected data were analyzed in SPSS V.16. To compare quantitative data t-student test was used and data Fisher exact test and χ^2 test were applied for qualitative data.

Results

In this retrospective case-control study, EBV genome was investigated in 79 samples of paraffin embedded breast cancer tissue including 67 cases (15.5%) of IDC, 8 cases (6.2%) of ILC, two cases (1.5%) of MC, 1 case (0.8%) of DCIS and 1 case (0.8%) of IDC – ILC and 51 sample of Fibroadenoma by Nested-PCR which all samples belonged to female patients.

The maximum and minimum ages in patients group were 22 and 75 years old, and in control group were 17 and 54 years, respectively. Statistical analysis between two age groups showed significant differences (P<0.05). From 67 samples of IDC, 24 samples (35.8%) were grade 1, 33 samples (49.3%) were grade 2 and 10 cases (14.9%) were grade 3 (Table 2).

Table 2. Characteristics of breast cancer samples infected with EBV compered to patient's age.

No	Type of cancer	Grade	Age (year)	Score
1	IDC	II	40	6
2	IDC	II	49	6
3	IDC	III	61	8
4	IDC	II	69	7

In all 130 samples, 96 samples had DNA of beta globin gene (individual primer as internal control) which was suitable for amplification via PCR method. Among them, 55 (57.3%) and 41 samples (42.7%) belonged to breast cancer and fibroadenoma, respectively. Regarding individual primers, 55 samples of breast cancer tissue had suitable quality to perform Nested-PCR. Of these, 4 samples (7.3%) had bands belonging to EBV. However, one (2.4%) had a band which belonged to EBV in 41 suitable tissue samples of control group for Nested-PCR. Prevalence of EBV infectivity in breast cancer was 7.3% and in Fibroadenoma was 2.4% in which there was no significant difference (P>0.05) (Table 3).

Table 3. Abundance of EBV-infected samples in suitable tissue samples for Nested-PCR in all types of available breast cancer samples.

Type breast cancer	IDC N=45	ILC N=6	IMC N=2	DCIS N=1	IDC.ILC N=1	P.V
Positive status	4(8.9%)	-	-	-	-	>0.05
Negative EBV-contaminated	41(91.1%)	6(100%)	2(100%)	1(100%)	1(100%)	

The only breast fibroadenoma sample infected with EBV was found in a 69 year old woman. All four breast cancer samples containing EBV were IDC type. Three cases (75%) were grade 2, and one sample (25%) was grade 3, so there was not significant difference in statistical analysis (P>0/05). The mean ages of cancer samples infected with and without EBV were 54.75 +12.8 and 47.69+12.5, respectively, and there was no significant difference in statistical analysis (P>0/05).

Discussion

Breast cancer incidence rate has been reported differently in the world. The highest rate was in the Northern Africa and Western Europe with age standardized rates (ASR) 123.6 and 84.6 in one hundred thousand people, respectively (34). In our study, EBV genome was investigated in 79 samples of paraffin embedded breast cancer tissue including 67 cases (15.5%) of IDC, 6.2% ILC, 1.5% MC, 0.8% DCIS and 0.8% IDC –ILC and 51 sample of fibroadenoma by Nested-PCR ,which all samples belonged to females. Modest incidence rate has been found in Mediterranean and Southern American countries (about 46.100000) and there are the lowest incidence rate in North Asia (25.100000) and central Asia (2.8.100000) too (36) . The prevalence of breast cancer is low in Asia, however, its rate of mortality in some Asian countries is more than that of the Western countries (37). The possible causes are changes in behavior and life style (38). In Iran, breast cancer is the most common cancer among women

(39, 40). It comprises 24.4% of all cancers (41) and the crude incidence rate was 17.81 and age-world-standardized incidence rate (ASR) of 23.65 per 100,000 in 2006 (40). For many years, the relation between EBV and cancer was limited to the presence of this virus in nasopharyngeal carcinoma and inflammatory cells (42). Although different factors are involved in breast cancer pathogenesis, its molecular fact has not been recognized yet (43). Trabelsi et al. considered presence of EBV in tumor cells and lack of them in non-tumor cells (natural cells) and in inflammatory cells for cause of breast cancer (44). Probably there is a relationship between infection with primary EBV in young adulthood and increased risk of breast cancer (45).

In this study using specific primer in 55 qualified samples of breast cancer tissue for Nested-PCR, 4 samples (7.3%) were found with EBV bands, while in 41 qualified samples of control group for Nested-PCR, one sample of fibroadenoma (2.4%) showed the EBV band. According to latter data, no statistical significant difference was noted. Thus, our area is a low risk EBV-infected region while other studies conducted in different part of the world showed different results. Our data were compatible with other low risk EBV-infected regions such as a study in the USA–(prevalence rate of 7%) (46) and another in Germany (prevalence rate of 6.8%) (47). In the USA a prevalence rate of 2% has also been reported (48).

EBV may exist during the early phase of malignancy in breast epithelial cells. But it will vanish through the trend of malignancy. On the other hand, presence of EBV in final step of tumor growth would induce severe oncogenic features such as invasion, angiogenesis and metastases (49, 50). These changes could cause more invasiveness of the subgroups of tumor cells (51). Due to the high prevalence of breast cancer, even a limited number of breast cancers having EBV are important (47) .The data showed no relation between EBV, patient's age and type of breast cancer.

Bonnet *et al*. also found similar results in lack of relation between EBV and tumor histology and other prognostic factors such as age at the time of diagnosis, tumor size and menopause, while in our study there was no association between tumor grade and presence of EBV. In addition, the ratio of positivity for EBV was significantly associated with higher grade cancer (52). The abundance of EBV-infected breast cancer in different places of the world is indicated in Table-4. Highest infection rate was reported in Japan (66%); however, just three cancer tissue samples were studied of which two had EBV-DNA (53). After Japan, Ukraine, France, some parts of the USA, and Taiwan also showed high prevalence rate (26, 36, 45, 52-54).

Table 4. The prevalence of EBV-infected breast cancer samples in different parts of the world.

Country	Reference number	Percentage (%)
Japan	54	66
Ukraine	55	54
France/Ukraine	53	51
France	26	46
USA	36	45.8
Taiwan	46	45.2
USA	34	45
USA	33	42
Ukraine	56	40
USA	57	36.36
Egypt	56	25
France	53	13.89
Iran	present study	7.3
USA	47	7
Germany	48	6.8
USA	49	2
Iran	58	0

Similarly, Fawzy *et al*. showed an association between EBV and some invasive breast cancers in Egyptian women and may play a role in their etiology (55).

Conclusion

Compared to other studies performed throughout the world we found north of Iran as a region with low-prevalence of EBV-infected breast cancer. This could be due to geographic differences, and genetic and environmental factors that affect the rate of this virus in different types of cancers especially breast cancer. Further studies in different regions and ethnics in Iran are recommended to verify the role of EBV in breast cancer.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

TZh and NAR developed the original idea, the protocol, technical, and material support (as administrators).

NF contributed to the study by interpreting and analyzing the data. NA participated in the study by writing, revising and constructively supervising the whole manuscript for important intellectual content. PM drafted the manuscript, and provided the abstract.

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