Original Article

Two Oncoviruses of HPV and EBV in Breast Cancer: An Iran-based Study

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ABSTRACT

OBJECTIVE: To answer the question of whether viral infections such as Human papillomavirus (HPV) and Epstein-Barr virus (EBV) are involved in the occurrence of breast cancer or not.

METHODOLOGY: In this case-control study, 50 paraffin-embedded breast samples (FFPE) and 50 non-cancerous FFPE samples were prepared from patients referred to Abadan hospitals from 2018 to 2021 to identify the genome of HPV and EBV by polymerase chain reaction (PCR). Data were analyzed using SPSS 15 with P-values of 0.05. Confirmed breast cancer biopsy samples with no medical history of chemotherapy and/or radiotherapy, the patient not being pregnant at the time of sampling, not using anti -cancer treatments, and not suffering from systemic inflammatory diseases such as rheumatoid arthritis. Patients of any age, with any breast cancer, and any size or stage of tumor were included without restrictions.

RESULTS: Using PCR, HPV DNA was detected in 7/50 (14%) and 2/50(4%) of breast cancers and normal control, respectively. Moreover, 71.42% and 28.57% of HPV-infected persons in the patient's group were genotypes 16 and 18, respectively. All HPV-infected people in the control group were low-risk genotypes 26 and 53. Also, EBV DNA was found in 2/50 (4%) and 6/50 (12%) of breast cancers and control samples, respectively. Co-infection was not detected in samples.

CONCLUSION: Although our study does not provide substantial Evidence about the role of viral infection in the progressiveness of breast tumors, significant studies have shown the critical role of these infections in the progression of breast tumors.

KEYWORDS: Human Papillomavirus, HPV, Epstein–Barr virus, EBV, breast cancer.

INTRODUCTION

Factors such as obesity, excessive alcohol consumption, and hereditary occurrence of mutations in BRCA1 and BRCA2 genes play a role in breast cancer[1]. In 2020, the World Health Organization (WHO) reported more than 685,000 deaths from breast cancer. In Iran, breast cancer is the most common malignancy among women, with a standardized incidence rate (ASR) of 23.1 per 100,000 persons[2].

Although the cause of breast cancer remains unclear, several risk factors, including age, premature menstruation, late menopause, obesity, and estrogen and growth hormone status, are known to develop this disease[3]. Oncoviruses such as *Epstein–Barr virus* (EBV) and *high-risk human papillomaviruses* (HPV), particularly types 16, 18, and 33, can cause almost 20% of tumors[4]. Oncoproteins of E5 and E6/E7 (in high-risk HPVs)[5] and LMP1, LMP2A, and EBNA1[6] (in EBV) are associated with viral-correlated carcinomas. Also, Epstein-Barr virus-encoded small RNA (EBER) can preserve the episomal form of the EBV DNA to increase the anti-apoptotic effect and malignant phenotype of B lymphocyte cells[6,7].

According to previous studies, HPV correlates with various carcinomas such as gastrointestinal tract, cervix-uterine, and breast cancer[8-10]. 90% of HPV-infected people are asymptomatic due to a healthy immune system, but long-term HPV infections can lead to malignant disease[10]. Also, EBV has been diagnosed with nasopharyngeal Cancer, Burkitt's lymphoma, and even gastric and breast cancer[3].

The association between oncogenic viruses and breast cancer remains controversial. Therefore, this

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doi: 10.22442/jlumhs.2023.01033
Received: 06-04-2023 Accepted: 13-07-2023
Published Online: 24-07-2023

possible connection has led to an interest in the survey of oncoviruses in breast cancer. In this study, we evaluate the association of HPV and EBV with breast cancer in tissue biopsies of breast cancer patients.

METHODOLOGY

Study design and sampling:

In this case-control study, 50 paraffin-embedded breast samples (FFPE) and 50 non-cancerous FFPE samples were prepared from patients referred to Abadan hospitals from 2018 to 2021. Inclusion criteria include confirmed breast cancer biopsy samples with no medical history of chemotherapy and/or radiotherapy, the patient not being pregnant at the time of sampling, not using anti-cancer treatments, and not suffering from systemic inflammatory diseases such as rheumatoid arthritis. Patients of any age, with any breast cancer, and any size or stage of tumor were included without restrictions.

The average age of the participants was 49.5±10.98 years. According to the Japan Cancer Society and WHO classification guidelines, the histological types of breast cancers in the current study were 46 ductal, three lobular, and one mucinous.

DNA extraction

Sections of 5-10μm thickness were prepared in DNase- and RNase-free microcentrifuge tubes. All procedures were performed according to Francisco Aguayo's study[11].

Polymerase Chain Reaction:

For EBV detection, the Polymerase Chain Reaction (PCR) conditions were 95°C for 20 S, 56°C for 60 S, and 72°C for 60 S (45 cycles). The primer fragments used in this study were as follows: Forward primer: 5´-TCTTGAGGATCCGCTAGGATA-3' and Reverse primer: 5´-ACCGTGGTTCTGGACTATCTGGAT-3'. The amplified sequences in samples and controls were characterized by agarose gel electrophoresis.

To detect HPV genomes, the Nested polymerase chain reaction (nested PCR) was used with universal primers (MY09/11, GP5+/6+) for the L1 gene according to the condition PCR of G. Capra et al. [12].

Real-Time PCR technique for HPV genotyping:

Genotyping of the HPV DNA was performed by Real quality RQ-HPV HR/LR Multiplex Kit (Italy) according to the manufacturer's instructions for detecting high-risk and low-risk genotypes.

Statistical analysis:

The mean with standard deviation and number (Percent) were used to describe quantitative and qualitative variables. Also, multiple logistic regressions were used to identify the association between study variables and breast cancer. Data were analyzed using SPSS 15 with a p-value of 0.05.

Consent for publication form:

Samples of this study were obtained from women who lived in Abadan and were diagnosed with breast cancer in past years. Some of these people have died, and we cannot access them. Hospital laboratories stored these samples in a nitrogen tank (− 80 °C); therefore, we used these samples for further studies on viral infection and breast cancer.

RESULTS

The genome of HPV and EBV was found in breast cancer samples. DNA was extracted from 100 FFPE breast tissues. Using nested PCR for the L1 region, HPV DNA was detected in 7/50 (14%) and 2/50(4%) in breast cancers and control samples, respectively. Moreover, 71.42% and 28.57% of HPV-infected persons in the patient's group were genotypes 16 and 18, respectively. All HPV-infected people in the control group were low-risk genotypes 26 and 53.

Using PCR for EBV EBNA-1, EBV DNA was found in 2/50 (4%) and 6/50 (12%) in breast cancer (**Figure Ia**) and control (**Figure Ib**) samples, respectively. Co-infection was not detected in samples.

A non-significant association was found between DNAs of viruses and age (**Table I**) and breast cancer (**Table II**). The mean and standard deviation of age in breast cancer were 49.5±10.98, and in the control group, they were 38.97±14.67. About 14% of patients with breast cancer and 4% of the control group have HPV, which was insignificant (P=0.081). Also, about 4% of patients with breast cancer and 12% of the control group have EBV, which was insignificant (P=0.140). **Figure II** shows the distribution of HPV and EBV in different groups.

Using Multiple Logistic Regression, **Table II** shows the odds ratio between breast cancer and study variables. The table shows no association between HPV (odds ratio: 2.02; p=0.421) and EBV (odds ratio: 0.18; p=0.068) with breast cancer.

Figure I(A): Gel electrophoresis of samples PCR product of EBV DNA (494bp): DNA molecular ladder (100 bp), sample 1(S1), sample 2 (S2), positive control (P.C.), negative control (N.C.), (B) sample 1 (S1), sample 2(S2), sample 3(S3), DNA molecular ladder (100 bp), sample 4(S4), sample 5(S5), sample 6(S6), positive control (P.C.), negative control (N.C.).

Figure A:

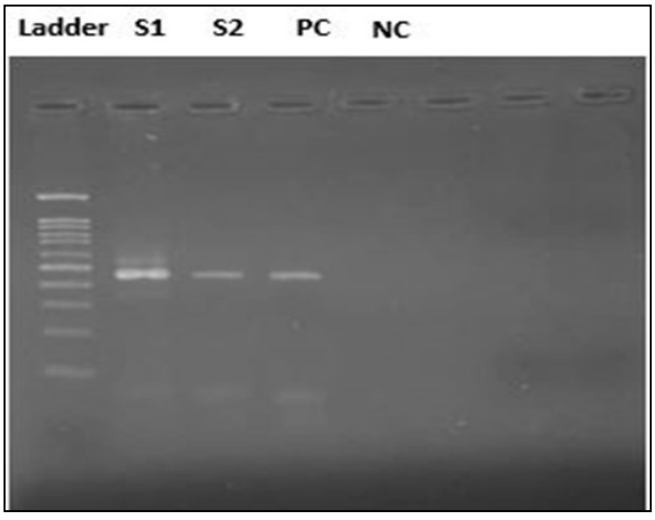

Figure B:

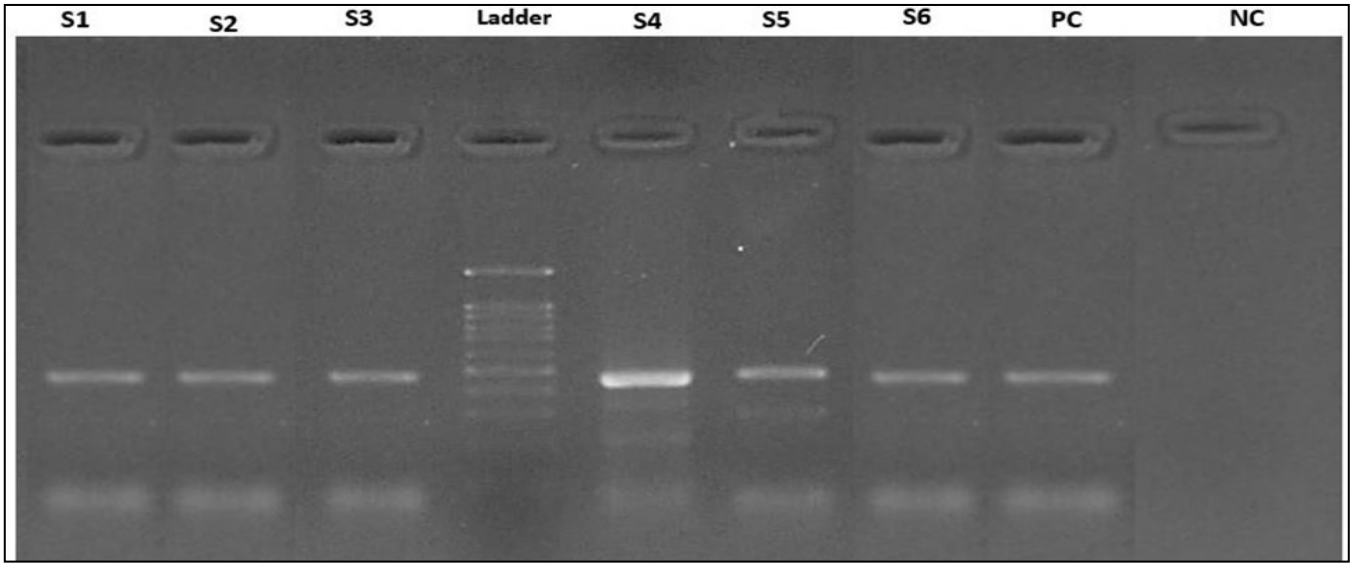

Figure II: Distribution of HPV and EBV in the case and control group

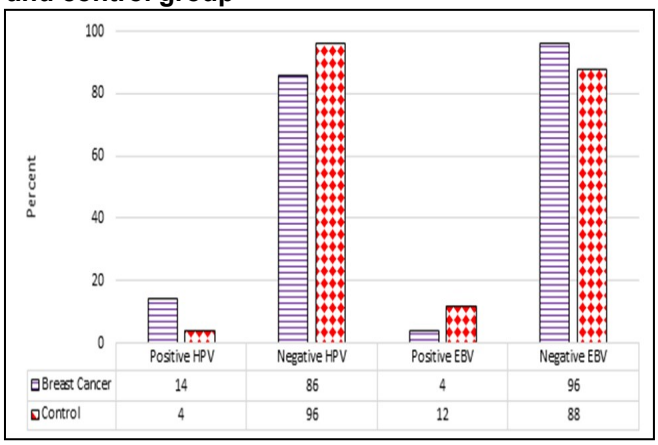

Table I: Distribution of Age, HPV and EBV between case and control groups

Variables		Breast Cancer	Control	P-value
Age		49.5±10.98	38.97±14.67	0.001
HPV	No	43 (86.0%)	48 (96.0%)	0.081
	Yes	7 (14.0%)	2 (4.0%)	
EBV	No	48 (96.0%)	44 (88.0%)	0.140
	Yes	2 (4.0%)	6 (12.0%)	

HPV: Human Papillomavirus, EBV: Epstein-Barr virus

DISCUSSION

The variable role of the viral infectious agents in cancers is controversial, as several studies suggest that the HPV[13], EBV[14], human cytomegalovirus (HCMV)[15], human herpesvirus type 8 (HHV-8)[16], and herpes simplex virus (HSV)-1 can stimulate Cancer malignant phenotype[17].

Table II:
Odds ratio between breast cancer and studies variables by using multiple logistic regression

Variables	Odd ratio	95% CI	p-value
Age	1.07	0.03 to 1.11	0.001
HPV	2.02	0.36 to 11.19	0.421
EBV	0.18	0.03 to 1.13	0.068

CI: confidence interval

About 80 percent of sexually active people are contaminated with one or more types of HPV, but high -risk HPVs are the most common strains associated with about 70% of several cancers[18]. Also, EBV DNA can be found in breast cancer[11,19].

Interestingly, in this study, the frequency of EBV in healthy controls was higher than in cancerous tissue, as reported by Kalkan A et al.[20]. These findings refer to the 'hit and run' mechanism that results in lost EBV DNA during cancerous cell division[21]. Furthermore, the frequency of EBV presence is high in normal populations. Since our project was a retrospective study, we did not check the serological status of participating women.

In addition, this controversy can be due to the duration of the fixation process of FFPE specimens and the use of conventional PCR assay[22]. Mofrad MG 2020[22] demonstrated that the detection of EBV by PCR technique in the FFPE sample is affected enormously by the fixation duration. On the other hand, PCR cannot detect EBV in infiltrating lymphocytes of cancer cells. To overcome this limitation of PCR, tumor cells separate from the neighbouring lymphocytes using laser capture microdissection (LCM) to affirm the localization of the viral DNA in the cancer cells by PCR[23]. Therefore, we recommend a study with larger sample sizes from different geographical areas and

using sensitive techniques on fresh biopsy specimens to clear the role of EBV in breast cancer.

In addition, HPV infection was higher in patients compared to the normal group (14% vs. 4%), with no significant relationship between HPV infection and breast cancer (P=0.081). The breast sample seems to have a low HPV load[24], so sensitive methods can be used to confirm HPV DNA.

On the other hand, of 7 positive HPVs in patients, 71.42% were genotype 16 (5 samples), 28.57% were genotype 18 (2 samples), and all HPV-infected people in the control group were low-risk genotypes 26 and 53. Our finding is consistent with the results of other geographical regions[25,26], but contrary to ours, several studies failed to detect HPVs in breast tissue samples[27-29].

CONCLUSION

Dozens of articles have been published, some of which support the link between viruses and breast cancer, while others have rejected the link. The discrepancy between studies throws up many questions on geographic/socioeconomic differences in viral infection prevalence, detection methods and types of tumors that require further studies; therefore, molecular, biological and epidemiological studies with larger sample sizes are needed to clarify the relationship between viral infection and breast cancer.

ACKNOWLEDGEMENTS

The article's authors are highly grateful for the valuable assistance of the Abadan University of Medical Sciences staff.

Ethical Statement: Abadan University of Medical Sciences Iran, ERC Letter No. I.R.ABADANUMS.REC.1398.013.

Conflict of Interest: No conflicts of interest.

Financial Disclosure / Grant Approval: No funding agency was involved in this research.

Data Sharing Statement: The corresponding author can provide the data proving the findings of this study on request. Privacy or ethical restrictions bound us from sharing the data publically.

AUTHOR CONTRIBUTIONS

Fani M: Manuscript writing, laboratory data collection
Sadooni R: Laboratory data collection
Nejad MH: Laboratory data collection
Mirzavieh NN: Laboratory data collection
Mobarak S: Laboratory data collection
Pakzad R: Analysis and interpretation of data
Erfani Y: Analysis and interpretation of data
Salmanzadeh S: Analysis and interpretation of data
Abbasi S: Conceptualization, designing the study, drafting the initial and final version of the manuscript

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