ORIGINAL ARTICLE

Presence of Human Papillomavirus and Epstein-Barr Virus in Breast Cancer Biopsies as Potential Risk Factors

Marla Ladera¹,*, Andreina Fernandes¹, Marco López², Adriana Pesci-Feltri², Maira Ávila¹ and María Correnti¹

¹Laboratory of Molecular Genetics, Instituto de Oncología y Hematología; ²Gynecology Department, Mammary Pathology Unit, Hospital Universitario de Caracas; Caracas, Venezuela

Received for publication: 8 October 2016; accepted for publication: 25 February 2017
Available online: 4 August 2017

Abstract

Introduction: Breast cancer represents the leading cause of cancer-related death in the Venezuelan female population. Many risk factors favoring its appearance have been reported; however, human papillomavirus and Epstein-Barr virus have been associated in the past few decades as potential risk factors in the development of this malignancy. Objective: To detect the presence of human papillomavirus and Epstein-Barr virus in breast cancer biopsies in order to establish a possible link between infection with these viral agents and the development of this pathology. Methods: Fresh biopsies were collected from patients with breast cancer and patients with breast benign pathology attending the Hospital Universitario de Caracas for surgery. Human papillomavirus detection was made using the INNO-LIPA® HPV Genotyping Extra commercial kit (Innogenetics), and Epstein-Barr virus genome was detected with Epstein-Barr Virus BMLF1 commercial kit (Maxim Biotech, Inc.). Results: 63.6 and 13.6% of breast cancer cases were positive for human papillomavirus and Epstein-Barr virus DNA, respectively, whereas the benign pathology samples had 4.5% positivity for each one of the viruses; 42.90% of breast cancer samples had mixed infection with low and high oncogenic risk human papillomavirus genotypes. Conclusion: We can suggest that human papillomavirus and Epstein-Barr virus are important risk factors for breast cancer; however, studies allowing for the role of these viruses in the development of the disease to be elucidated are required.

KEYWORDS
Breast cancer; Human papillomavirus; HPV; Epstein-Barr virus; EBV; Risk factors

*E-mail for correspondence: marla.ladera92@gmail.com (M. Ladera)

doi:10.24875/j.gamo.17000018
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INTRODUCTION

Breast cancer is the second most common cancer in the world. In 2012, the International Agency for Research on Cancer (IARC) estimated 1.6 million new cases of breast cancer, accounting for 25% of all types of cancer diagnosed that year. In Venezuela, according to the People’s Power Ministry for Health (MPPS – Ministerio de Poder Popular para la Salud) Mortality Yearbook, breast cancer was the leading cause of oncological death in the female population in 2012, with 2,067 deaths being reported, and this pathology turning into an alarming public health problem and that, although it can affect males, current male mortality figures in the country do not exceed 10 annual cases.

The probabilities for developing breast cancer increase or decrease according to the number of concurring risk factors, which include the environment, genes, and lifestyle. Currently, there are well known risk factors associated with breast cancer; however, in most women, identifying a particular factor related to the development of the disease is not possible. In the past few decades, the action of a group of tumor-producing viruses has been considered to be an important factor in the development of cancer in both experimental and human models. The human papillomavirus (HPV) is the etiologic agent of cervical cancer, and the Epstein-Barr virus (EBV) is mainly associated with nasopharyngeal carcinoma and Burkitt’s lymphoma. These two viruses have been frequently associated with the development of breast cancer owing to their oncogenic role, either by the expression of viral genes that are similar to cell genes, or by the expression of proteins that are able to alter the cell cycle.

Human papilloma virus is a sexually transmitted virus and member of the Papillomaviridae family; its genome is composed of double-stranded, circular DNA of approximately 8,000 bp. This virus has special affinity for epithelial cells, and infects uterine cervix stratified squamous epithelium basement membrane cells; however, expression and replication of its genes depends on normal epithelium differentiation. This dependence of the virus on host cells, together with the expression of viral oncoproteins, drive the cell to a proliferative state without cell lysis occurring. The E6 and E7 oncoproteins are able to interact with p53 and pRb cell proteins, respectively, thus affecting their functions on cell-cycle regulation and driving to permanent cell immortalization. In addition, HPV genome integration interrupts and eliminates E2 open reading frame, with expression of this viral gene being lost. This way, integration of the viral genome and concomitant loss of E2 expression could be an important step in the carcinogenic process resulting from the altered expression of viral genes E6 and E7, given that the E2 protein acts as viral replication negative control. Based on this affinity of HPV for epithelial cells and its important capacity to lead to the development of cancer, numerous studies have investigated the presence of HPV in breast tissue epithelial cells, with some works having reported little or no evidence, while others have reported important infection figures in breast cancer patients, additionally proposing possible routes for HPV infection in breast tissue.

In turn, EBV is an enveloped virus, member of the Herpesviridae family, which is constituted by a linear, double-stranded DNA genome of approximately 172,000 bp. It is a virus with great affinity for B-cells and oropharyngeal epithelial cells, which are main route of infection with the virus, with two types of replication cycles having been able to be established: a lytic cycle, and another of latency in the host cell. Although EBV is frequently associated with human lymphoid neoplasms in immunosuppressed patients, several studies have demonstrated that there is also a possible association between EBV and the development of malignancy epithelial cells such as breast tissue, mainly based on some observations that support this hypothesis: (i) high incidence of male breast cancer in EBV-endemic Mediterranean countries; (ii) development of EBV-associated lymphomas at the level of the breasts; and (iii) morphological similarities between breast medullary carcinomas and nasopharyngeal carcinomas. The most specific evidence of an association of EBV with breast cancer has been the identification of EBV gene sequences in breast tumors. On the other hand, the expression of EBV latent protein expression has also been assessed in breast cancer cell lines in vitro and in vivo, as well as the oncogenic capacity of its viral proteins. However, some studies have failed to find any relationship between EBV and breast cancer.

These evidences allow for a possible relationship between breast cancer and infection, either with EBV or HPV, to be proposed, based on which these viruses can be regarded as risk factors in the development of this disease that causes many deaths in the female population worldwide. Based on this, the purpose of this work was to assess the presence of HPV and EBV in fresh biopsies of Venezuelan female patients with breast cancer as possible risk factors associated with this pathology.

MATERIALS AND METHODS

Patients

From April 2014 through May 2015, 44 Venezuelan patients attending the outpatient clinic of the Caracas University Hospital (HUC - Hospital Universitario de Caracas) Gynecology Department Breast Pathology Unit (UPM - Unidad de Patología Mamaria) were prospectively assessed. Each patient was invited to participate in the study after being briefed on the study design and protocol, with each one signing a HUC Bioethics Committee-approved informed consent form. Patient selection was made under the following selection criteria: patients diagnosed with stage 0, I, and II breast carcinoma, and those patients diagnosed with stage III and IV breast carcinoma who, for intrinsic reasons, did not receive neoadjuvant therapy, as well as patients diagnosed with benign breast pathology were included. Patients diagnosed with autoimmune disease or any other cancer not related to the primary tumor, pregnant patients, patients receiving neoadjuvant therapy, and those not accepting to participate in the study were excluded.

Genetic material extraction and quality assessment

Genetic material extraction was carried out with the PureLink™ Genomic DNA commercial kit (Invitrogen), following
the manufacturer’s specifications. The DNA quality of all samples was assessed following the BIOMED-2 protocol by Van Dongen, et al.\textsuperscript{26}. The reaction mixture was prepared using 0.1 µl of dNTP (100 mM), 2 µl of each primer (100 µM), 6.5 µl 10X buffer, 2 µl MgCl\textsubscript{2} (50 mM), 0.6 µl Taq polymerase, and 28.8 µl nuclease-free H\textsubscript{2}O, for a final volume of 50 µl. Amplification conditions were at 95 °C, 35 30-second cycles at 45 °C, 40 seconds at 60 °C, and 40 seconds at 70 °C, and a final amplification of 15 seconds at 70 °C.

**Human papilloma genome detection and typing**

Detection and genotyping of HPV 28 genotypes in breast cancer biopsies was carried out with the INNO-LIPA\textsuperscript{®} HPV Genotyping Extra kit (Innogenetics), following the manufacturer’s recommendations. This test involves an assay with immobilized probes in nitrocellulose strips, based on a reverse hybridization principle, designed to identify 28 different HPV genotypes by means of the detection of HPV genome L1 region specific sequences. This assay uses a series of SPF10 primers for higher amplification sensitivity of most more clinically relevant HPV genotypes.

**Epstein-Barr virus genome detection**

Epstein-Barr virus detection was made with the Epstein-Barr virus BMLF1 commercial kit (Maxim Biotech, Inc.), following the manufacturer’s specifications. The reaction mixture conditions were the following: 40 µl of master mix (Buffer, dNTP, MgCl\textsubscript{2}), 0.2 µl Taq polymerase, and 10 µl of sample DNA were used to obtain a final volume of 50.2 µl. Amplification conditions were one minute at 96 °C, 35 one-minute cycles at 94 °C, one minute at 58 °C, one minute at 72 °C, and a 10-minute final amplification at 72 °C. Only those samples where a 265 bp band was observed were regarded as positive.

**Agarose gel electrophoresis**

The PCR amplification products were visualized by means of agarose gel electrophoresis at 2% with Invitrogen 10X TBE 1X stock buffer (1.0 mM base Tris, 0.9 mM borate and 0.01 mM EDTA, pH 8.0) and were stained with SYBR\textsuperscript{®} SAFE (Invitrogen). Photographic registration was carried out with a ChemiDoc\textsuperscript{™} imaging system (Bio Rad).

**Statistical analysis**

Descriptive analyses were performed using central tendency and dispersion measures (mean, median, standard deviation) for continuous variables, and frequency analysis and contingency tables for discrete variables. The chi-square test was used to assess dependence of variables with the Microsoft Excel Office 2010 program. P-values ≤ 0.05 were considered to be statistically significant.

**RESULTS**

A total of 22 breast cancer patients and 22 benign breast pathology patients were included. Clinical characteristics of the study population are shown in table 1, where mean age, mean menarche, mean full-term pregnancies, mean number of partners, and use of oral contraceptives (OC) are indicated. Of total breast cancer samples, 63.6% corresponded to patients with stage II tumors, followed by stage I with 18.2%. With regard to histopathological diagnosis, collected samples of breast cancer tumor corresponded to ductal carcinoma in situ (DCIS), infiltrating ductal carcinoma, infiltrating lobular carcinoma, and infiltrating papillary carcinoma, with infiltrating ductal carcinoma being the most common in the group with 68.2%.

Once the genetic material was extracted from the study biopsies, its quality could be corroborated by identifying five constitutive expression genes in order to ensure the genome was in optimal conditions. All samples amplified the target genes, since the collected specimens were fresh biopsies preserved at -80 °C, without the need to be embedded in paraffin, thus avoiding possible DNA fragmentation during the processing of the sample to prepare paraffin blocks, which complicates viral DNA detection.

Subsequently, detection and genotyping was carried out, with 14 out of 22 (63.6%) breast cancer samples being found to be positive for HPV DNA, whereas in healthy tissue samples, HPV DNA was only detected in one sample, accounting for 4.5% of all benign pathology evaluated patients (Table 1). Then typing was performed and we found that among the 14 positive samples, the most common types were 6 and 11, (27.27% each), followed by type 16 (21.21%). With regard to the HPV-infected patient with benign pathology, the high oncogenic risk HPV-33 viral genotype was identified. Figure 1 shows a bar graph with the frequency of each one of the HPV genotypes found in the patients with breast cancer.

| Table 1. Clinical characteristics of patients diagnosed with breast cancer and benign pathology |
|---------------------------------------------|---------------------------------------------|
| **Breast cancer**                          | **Benign pathology**                        |
| Age mean                                   | 58 years (range 30-86)                      |
| Menarche mean                              | 12.5 years (range 9-17)                     |
| Pregnancies mean                           | 3.14 pregnancies (range 0-9)                |
| Partners mean                              | 1.85 partners (range 1-4)                   |
| Use of OC                                  | 45.5%                                       |
| HPV presence                               | 63.6%                                       |
| EBV presence                               | 13.6%                                       |
| **Benign pathology**                       |                                             |
| Age mean                                   | 29.4 years (range 14-50)                    |
| Menarche mean                              | 12.5 years (range 9-17)                     |
| Pregnancies mean                           | 1.32 pregnancies (range 0-6)                |
| Partners mean                              | 1.73 partners (range 0-5)                   |
| Use of OC                                  | 72.7%                                       |
| HPV presence                               | 4.5%                                        |
| EBV presence                               | 4.5%                                        |

EBV: Epstein-Barr virus; HPV: human papilloma virus; OC: oral contraceptives.
Clinical data of the assessed breast cancer patients are consistent with those obtained in previous publications, where the age range of breast cancer-affected patients has been reported to be between 45 and 65 years in Venezuela and between 50 and 80 years in European and North American countries, whereas benign pathologies are associated with early female ages all over the world. The high incidence of benign lesions in young women has been proposed to be a result of the hormone load being higher at younger ages, where hormone stimulus during the reproductive cycle drives to an increase in cell mitotic activity, with the risk for developing benign pathologies on that site increasing if young patients have hormone imbalances, whereas in middle-aged and elderly patients, the presence of benign pathologies is proportionally lower with higher numbers of full-term pregnancies, which possibly gradually decreases the hormone load.

In this study, the presence of HPV was detected in 63.6% of breast cancer samples, while only 4.5% of benign pathology tissue biopsies were positive for this virus. This detection frequency is within the previously published worldwide range, with a prevalence ranging from 10-86%. Among the works by Akil, et al. in 2008 and Antonsson, et al. in 2011 detected 61.06 and 50% of HPV DNA-positive cases by means of multiplex PCR and assembly PCR, respectively.

In Latin America, the rate of HPV infection in breast cancer is low. Positivity has been reported to range between 8-40%. Therefore, our work is the first to report high frequency of HPV in malignant breast tissue of Venezuelan female patients. However, some studies have failed to detect HPV in breast tumor or normal tissue. This difference in published reports can be attributed to the number of assessed samples, differences in methodology, and sensitivity of the employed methods, such as the use of different primer kits, as well as the type of sample used.

When identification of HPV viral genotypes was made, we found that all 14 positive samples showed single and mixed infections with oncogenic high-risk genotypes 16, 18, 52, and 56, and oncogenic low-risk genotypes 6 and 11. In malignant tumors, the most common high-risk genotype was 16 (21.21%), and in the case of low-risk genotypes, the types found were 6 and 11 (27.27% each). In the patient with benign pathology infected with HPV, viral genotype 33 of oncogenic high-risk, was identified. These identified genotypes are consistent with those reported at the global level, where the most commonly detected oncogenic high-risk genotype is HPV-16 in between 20 and 90%.

When viral genotypes were grouped according to their oncogenic risk, we found that 28.57% of breast cancer patients had single infections with high- or low-risk genotypes, and 42.90% of HPV-positive patients showed coinfection with both genotype groups.

High-risk genotypes 16 and 18 are associated with an accelerated growth of cervical epithelial cells, since they tend to integrate into the genome and drive to uncontrolled cell proliferation owing to an inhibition of cell proteins p53 and pRb function. In this work we found that out of 57.1% of HPV 16 and/or 18-infected patients, 75% of them had elevated expression of cell proliferation Ki67 marker reported.
on medical records, whereas in the rest of the patients infected with other viral genotypes (6, 11, 52, 56), only one out of six patients had Ki67 over-expression reported, which suggests that the presence of high-risk genotypes (16 and 18) can drive to uncontrolled cell proliferation in breast tissue.

Ever since the presence of EBV was first reported, the number of studies focused on the oncogenic potential of this and other viruses has been progressively increasing. In this sense, new investigations have been carried out associating this virus with other types of lymphomas and several types of carcinoma, such as gastric and lung carcinoma and, recently, breast carcinoma17,37,38. These associations are based on the type of cells EBV is able to infect, which include B-cells and epithelial cells. In addition, most these tumors are characterized by the presence of multiple viral genome extra-chromosomal copies and expression of EBV-encoded latent genes, which contributes to the malignant phenotype39.

In this study, the presence of EBV was detected in 13.6% of Venezuelan female patients with breast cancer, whereas in the studied patients with benign pathology, EBV DNA was detected in only one patient (4.5%). This result is below the values reported in other studies conducted at a global level, which range from 21-55% of EBV DNA positivity, depending on the type of technique employed for viral DNA detection4,19,40. In works using some type of PCR, as in our study, low infection rates were detected, as in the trial by Xue, et al. in 2003, who, using BZLF1 gene-specific reverse transcriptase PCR, detected viral DNA in 17% of breast cancer cases, and the work by Yahia, in 2014, who detected 11% of EBV DNA positivity in breast cancer cases using PCR with EBNA-1 gene-specific primers41,42.

In Latin America, very few studies have assessed the presence of EBV in breast cancer, and have reported a rate of infection ranging from 6-31%31,41. Differences found with regard to other studies may be due to EBV epidemiology variations according to the geographic region.

Therefore, with EBV being a co-factor in the development of several malignancies, including different carcinomas (nasopharyngeal carcinoma, gastric carcinoma)18, we can suggest that the presence of EBV in breast tissue tumors might be an important risk factor for breast cancer, owing to its oncogenic potential. However, it is important to take into consideration the genomic DNA load of the virus and the amount of assessed DNA in the samples, in order to avoid discrepancies in viral DNA detection.

With regard to co-infection with HPV and EBV in breast cancer, all three patients who had EBV DNA were found to also have HPV DNA detected, accounting for 13.6% of all assessed patients with breast carcinoma, with this co-infection rate being intermediate with regard to that reported in other studies at the global level, which ranges from 2.1 to 38%20,37. It is important to highlight that there are only few studies that have assessed the presence of this virus in South America. In Chile, Aguayo, et al.37 studied co-infection with EBV and HPV, and found 6.5% of EBV positivity in breast cancer samples.

To the best of our knowledge, this is the first study to assess the presence of both viruses in Venezuelan female patients with breast cancer. Even though there was no statistically significant relationship with regard to the presence of both viral agents, the biological behavior of viruses should not be disregarded.

CONCLUSIONS

The presence of HPV was detected in 63.6% of breast cancer patients, whereas EBV was detected in 13.6%. Even when there were no statistically significant differences, we can suggest that HPV and EBV might act as important risk factors in breast cancer pathogenesis, owing to their oncogenic characteristics. Our finding is mainly based on viral genome detection with the PCR and reverse hybridization technique, and confirmatory tests would therefore be required. In addition, further investigations are needed to determine the role of HPV and EBV in breast cancer etiology or progression, including viral load assessment for both agents and determination of HPV viral integration to the host genome, since this is known to be an important step in the development of carcinogenesis that affects important viral replication checkpoints. In addition, increasing the number of samples is necessary, which will allow for a clearer tendency to be observed with regard to the relationship between the presence of these viruses and the development of breast cancer.

DECLARATION OF INTEREST

The authors declare not having any conflicts of interests.

The authors thank the funding granted by research projects PII no. 2012001201 and FONACIT G2005000408.

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HPV and EBV in breast cancer


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