RESEARCH ARTICLE

Molecular detection of Epstein-Barr virus in breast cancer among Sudanese female population: a case-control study

[version 1; peer review: 2 not approved]

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Abstract

Background: Breast cancer is the most common cancer in women worldwide and in Sudan. Breast cancer occurs due to a multifactorial process and infection with an oncogenic virus has been recently investigated as a possible risk factor for breast cancer. For nearly two decades, studies have incriminated Epstein-Barr virus (EBV) in the etiology of breast cancer. However, the results are unconvincing, and their interpretation has remained a matter of debate. The aim of this study was to detect EBV in breast cancer biopsies obtained from Sudanese female patients.

Methods: A descriptive, hospital-based, case-control study, conducted at Faculty of Medical Laboratory Science, University of Khartoum, Khartoum, Sudan. Archival blocks were obtained from 115 patients with breast cancer and 115 controls during the period between November 2016 till March 2017.

Results: Among 115 breast cancer tissue specimens, EBV DNA was identified in 42/115 (36.5 %) samples and was not identified in 73/115 (63.5 %) tissue samples. The highest frequency of EBV detection was among 41–60 year-olds (23/42, 54.7 %), followed by 21–40 year-olds (12/42, 28.5 %) and 61–80 year-olds (5/42, 11.9 %). In the control group, the majority were diagnosed with fibroadenoma (70.4%), followed by fibrocystic changes (10.4%) and lactating changes (0.9%).

Conclusion: The data obtained in this study demonstrated that EBV was present in a high percentage of our study population; however, the exact role of EBV in Sudanese breast cancer needs to be studied more in depth.

Keywords

Breast cancer, Invasive Ductal carcinoma, Epstein-Barr virus

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Approval Status

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Any reports and responses or comments on the article can be found at the end of the article.

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Abbreviations

EBNA-4, Epstein-Barr virus nuclear antigen 4; EBV, Epstein-Barr virus; LMP1, latent membrane protein 1; MEC, mammary epithelial cell; PCR, polymerase chain reaction

Introduction

Epstein-Barr virus (EBV) was discovered in the early 1960s by direct electron microscopy and it infects up to 95% of the adult human population, primarily in their early life. It usually remains silent and doesn't cause any symptoms as the immune system doesn't treat the virus like an invader following the initial infection. However, infection can lead to severe manifestations in later life^{1,2}. This virus has been found to be the cause of many types of cancers including Burkitt's lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma, nasopharyngeal carcinoma, as well as Leiomyosarcoma arising in immunocompromised individuals^{3,4}. The main mechanism by which the virus can induce cell transformation from normal to malignant cells is not yet understood and is still under investigation, although some researchers have related this to EBV viral protein effects⁵⁻⁸.

Breast cancer is one of the most frequent types of cancers and is the leading cause of death in females globally⁹. Several factors can promote the development of breast cancer, including infections, especially oncogenic viruses that have been proven to be possible risk factors for the development of the cancer. These viruses include mouse mammary tumour virus (MMTV, causes breast cancer in mice), EBV and human papilloma virus (HPV)¹⁰⁻¹². EBV DNA was identified previously in several studies using different molecular based techniques, including sequencing, polymerase chain reaction (PCR), *in situ* hybridization, immunohistochemistry and real-time PCR¹³.

In Sudan, breast cancer is a leading type of cancer in adults, along with leukemia, prostatic carcinoma, lymphoma and colorectal carcinoma¹⁴. Therefore, we aim in this study to detect EBV in breast cancer biopsies obtained from Sudanese female patients.

Methods

Study design

This is a descriptive, hospital-based, case-control study, conducted at Faculty of Medical Laboratory Science, University of Khartoum, Khartoum City, Sudan, during the period of November 2016 until March 2017.

Sample collection

Samples were collected from Soba Teaching Hospital and Military Hospital in Khartoum state, Sudan, during the period of November 2016 to January 2017. Archival blocks of 115 female patients diagnosed with breast cancer and 115 normal breast lesions (controls) were retrieved for the detection of EBV. All archival blocks from female patients diagnosed with breast cancer were included in the study, with no further inclusion or exclusion criteria. For the controls, archival blocks from women with a breast lesion, not diagnosed as breast cancer, taken during the same period as the breast cancer patient samples were included. The control samples were not matched to the breast cancer patient samples. Analysis of these blocks were carried out during the period of February 2017 to March 2017.

Preparation of formalin-fixed paraffin-embedded tissue

Formalin-fixed paraffin-embedded blocks (FFPB) were cut at a thickness of 10 µm using a rotary microtome and the ribbons of the cut tissues transferred into 1.5 ml eppendorf tube using sterile forceps. The samples were deparaffinized by adding 1 ml of xylene to each sample and incubating for 30 minutes to allow the removal of the wax from the tissue sections. This step was repeated twice. The tissue was rehydrated using a series of ethanol washes, using 95%, 80% and then 70% ethanol for 15 min for each.

DNA extraction and assessment of DNA quality

The DNA was extracted using the phenol-chloroform method with the aid of lysis buffer that composed from Sodium dodecyl sulfate (SDS) and proteinase K (40 µl) to get rid of proteins, as previously described¹⁵⁻¹⁷. To purify the DNA from the lysate, buffer saturated phenol was used. Then, in order to precipitate the DNA, 3M sodium acetate and isopropanol were added and this was spun in a centrifuge at 16000 rpm for seven mins to pellet the nucleic acid. This step was followed by washing in 70% ethanol to get rid from the salts and centrifugation at high speed to reconstruct the pellet again.

DNA was resuspended in distilled water, quantified and stored at -20 °C. The final extracted DNA was checked for purity and quantity using a Nanodrop 1000 spectrophotometer. If the 260/280 nm absorption ratio was between 1.8-2, the DNA was considered to be pure. Purified DNA was used for PCRs.

Molecular identification of EBV

To determine if the EBV DNA was present in our samples, DNA was amplified for detection of the EBV LMP-1 gene using forward 5'- CCG AAG AGG TTG AAA ACA AA-3 and reverse 5'- GTG GGG GTC GTC ATC ATC TC-3 primers. PCR was performed in a final reaction volume of 25µl, with 1 µl of template DNA, using iNtRON's Maxime PCR PreMix Kit (i-Taq, Korea; Catalog No. 25025) according to manufacturer instructions. The following PCR conditions were used: 4 min at 95°C; then 35 cycles consisting of a denaturing step at 94°C for 1 min, an annealing step at 60°C for 1 min and a polymerization step at 72°C for 1 min; followed by a single incubation step for 10 min at 72°C. PCR was performed on an Aeris Thermocycler (AERIS-BG096, Esco Micro Pte.Ltd, Singapore).

Analysis of PCR results

The amplification product of 131 bp was separated on a 2% agarose gel stained with ethidium bromide and visualized under UV light using the UVCI-1100 gel imaging system (Major Science, UVCI-1100, Saratoga, CA, USA). A 100 bp DNA ladder was used and a band of 131 bp was considered as a positive (Figure 1).

Statistical analysis

The statistical analysis was performed using SPSS (version 16.0) statistical software. Descriptive statistics were conducted to generate graphical and numerical summaries. The Chi-squared test was used to compare the frequencies of the categorical variables. A value of $p < 0.05$ was considered statistically significant.

Figure 1. Gel electrophoresis of PCR products. PCR products were loaded on 2% agarose gel containing ethidium bromide and visualized under UV light. Lanes 1 and 13 show the 100bp DNA ladder. Lanes 2 – 12 show different samples containing the PCR product of 131 bp.

Ethical statement

The study was approved by the faculty research board of the faculty of Medical Laboratory Sciences, University of Khartoum, Sudan. Written informed consent was obtained from patients for the use of their samples and publication of results.

Results

The age of the breast cancer patients ranged between 23 and 80 years and the mean age was 50.49 ± 9.50 years, while the control group age ranged from 25 to 72 years and the mean age was 37.65 ± 5.89 years¹⁸. Regarding histological type, 96 (83.5%) of the patients diagnosed with breast cancer had invasive ductal carcinoma, 18 (15.7%) had invasive lobular carcinoma and one (0.9%) had mucinous carcinoma. Regarding the clinical staging (according to the TNM staging of the American Joint Committee on Cancer), 10 (8.7%) were stage I, 13 (11.3%) were stage IIA, 15 (13%) were stage IIB, 17 (14.8%) were stage IIIA, 24 (20.9%) were stage IIIB and 36 (31.1%) were stage IV (Table 1). In the control group, the majority were diagnosed with fibroadenoma (70.4%), followed by fibrocystic changes (10.4%) and lactating changes (0.9%).

Among the 115 breast cancers tissue specimens, EBV DNA was identified in 42/115 (36.5 %) samples and was not identified in 73/115 (63.5 %) tissue samples (Table 2). In the control group, EBV was detected in 101/115 (87.8%) and only 14/115 (12.2%) tested negative for the presence of the virus. The Chi-square test showed that EBV has a significant effect on breast cancer with a p-value of 0.001.

The highest frequency of infection was seen in the age group 41–60, with infection found in 23/42 (54.7 %), followed by age ranges 21–40 (12/42, 28.5 %) and 61–80 (5/42, 11.9 %).

Notably, with regard to EBV infection and staging of breast cancer, by far the majority of infections were detected among stage IV (11/42, 26.1%), followed by stage IIIA (10/42, 23.8%), and stage IIIB (8/42, 19.0%), although these results were shown to be statistically insignificant (Table 3).

Discussion

Breast cancer has been proven to be a leading cause of death amongst women in the western world after lung cancer. Its incidence has been confirmed to be higher in females than males, and the number of cases increase with age. Early case detection has improved due to the promotion of screening procedures and diagnosis^{19,20}. In Sudan, breast cancer still accounts for many cases of cancer among women. In 1959, breast cancer was found to account 22.9% of all cancer cases. The bulk of those patients were females within the menopausal age, with disease clinically progressing in a similar pattern as in European ladies^{21,22}. In 2010, an updated study of breast cancer prevalence in Sudan by Intisar and colleagues found a prevalence of 25.1 per 100,000²³.

For approximately twenty years, studies have been conducted that have aimed to identify the association between EBV and breast cancer. The first report of an association between EBV and breast cancer was from a study that carried out screening of blood

Table 1. The distribution of study population by age, histological type and staging.

Characteristic	Percentages
Age range	
21–40	22 (10.1%)
41–60	77 (67.0%)
61–80	16 (13.9%)
Histological subtypes	
Invasive ductal carcinoma	96 (83.5%)
Invasive lobular carcinoma	18 (15.7%)
Mucinous carcinoma	1 (0.9%)
Stage	
Stage I	10 (8.7%)
Stage II A	13 (11.3%)
Stage II B	15 (13%)
Stage III A	17 (14.8%)
Stage III B	24 (20.9%)
Stage IV	36 (31.3%)

Table 2. The distribution of Epstein-Barr virus (EBV) among patient and control groups.

Patients	Controls		Total
	Negative	Positive	
Negative	59	14	73
Positive	42	0	42
Total	101	14	115

(Chi square test; p value = 0.001).**Table 3.** The distribution of the study population by staging and Epstein-Barr virus (EBV) infection.

Staging	EBV		Total
	Positive	Negative	
Stage I	2	8	10
Stage II A	7	6	13
Stage II B	4	11	15
Stage III A	10	7	17
Stage III B	8	16	24
Stage IV	11	25	36
Total	42	73	115

(Chi square test; p value > 0.05).

and DNA of 91 patients with breast cancer by PCR in order to detect the EBV genome in 1995²⁴. Since that, other studies have confirmed the finding that EBV is associated with breast cancer^{25–33}. However, these results have been controversial since negative results were obtained in some studies and explanations of the results have been under discussion for several years^{25,34–38}.

Moreover, in 2016 Hu and colleagues queried whether the EBV can be associated with breast cancer development. NOD/SCID mice were used and their results suggested that EBV can infect primary human mammary epithelial cells (MECs) through CD21, leading to phenotypic variations consistent with transformation. These immortalized MECs infected with EBV cooperatively (with activated Ras) increase tumor formation *in vivo*, recapitulating a multi-step tumorigenesis in an established animal model³⁹.

A study was conducted in 2014 by Yahia and colleagues to study the relationship between breast cancer and the presence of EBV genome. Interestingly, this was the first study in Sudan to assess the role of EBV infection in the etiology of breast cancer. They used PCR and *in situ* hybridization to screen 92 breast cancer samples, aiming to measure the presence of Epstein-Barr nuclear antigen 4 (EBNA-4) and LMP-1. Their final outcome was statistically significant ($p = 0.0001$), demonstrating the presence of EBV genome in 49 (53.3%) and 10 (11%) patients by LMP-1 and EBNA-4 PCR, respectively¹⁰. This result is in agreement with our results, which showed that EBV DNA was identified in 42/115 (36.5 %) samples and was not identified in 73/115 (63.5 %) tissue samples.

Notably, the incidence of EBV among Iranian females with breast cancer has been found to be low compared to our findings. In 2016, Mohammad Izadeh and colleagues tested LMP-1 antigen expression in breast carcinoma using immunohistochemistry in 80 Iranian patients. LMP-1 expression was detected in 6 cases (7.5%) of breast carcinoma cases and these results were found to be statistically significant. LMP-1 expression was not detected in the normal breast tissue, taken from a site adjacent to the carcinoma, in all cases⁴⁰. Furthermore, another study from Iran, conducted by Kadivar and colleagues, to evaluate the presence of EBV in benign and malignant breast lesions using PCR and immunohistochemistry is also in disagreement with our results, as all breast cancer samples were negative for EBV, as were the control group samples⁴¹.

The present study verified the presence of EBV in 36.5% of the total breast cancer samples, in agreement with the study conducted by Bonet and colleagues, in which 51 (51%) cases were found to be positive for EBV by PCR out of a total of 100 breast cancer cases²⁷.

However, this study has some limitations. The study did not use cohort techniques to assess whether EBV viral load had an effect on the prognosis of breast cancer and we could not use advanced techniques for visualization of the virus in the malignant cells due to time and budget constraints.

Conclusion

In conclusion, the data obtained in this study demonstrated that EBV was present in a high percentage of our study population; however, the exact role of EBV in Sudanese breast cancer needs to be studied more in depth.

Data availability

Underlying data

Harvard Dataverse: Replication Data for: EBV and Breast cancer Data among Sudanese patients. <https://doi.org/10.7910/DVN/94YOCV>¹⁸

EBV_and_Breast_cancer_Data.tab (diagnoses, PCR results and ages of patients and controls)

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

Grant information

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James Lawson

School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, Australia

Unfortunately the manuscript and the study have serious flaws.

The most important flaw is the lack of recognition of infiltrating lymphocytes in breast cancer specimens. These lymphocytes can be Epstein Barr positive and give false positive outcomes of PCR based studies.

This issue has long been recognised by others and is repeatedly referred to in the literature

This problem can be overcome in several ways as published.

In addition the results of the controls are not reported properly? See Table 2. This table does not make sense. This problem has been commented by a previous reviewer.

I am sorry but this project is not of sufficient quality to deserve indexing.

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

No

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 16 December 2019

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Sulma Ibrahim Mohammed

Department of Comparative Pathobiology, Purdue University Center for Cancer Research, Purdue University, West Lafayette, IN, USA

In the Abstract: Method: the age of the control group is far younger compared to patients (37 vs 50).

Result section - control group - only the histological type is provided. It should show the virus presence in tissue of patients with breast cancer compared to control without cancer - that does not make sense.

In the Methods: how are the tissues fixed and embedded in blocks and how long?

Results: Table 1 - describe only patients - why not control as well?

Table 2: does not make sense and is not clear.

In results sections: last paragraph state that "In the control group, EBV was detected in 101/115 (87.8%) and only 14/115 (12.2%) tested negative for the presence of the virus" that means the virus is present in the majority of control samples - which render the conclusion wrong.

Fig 1 is not necessary unless control samples are included as well.

Discussion: the author should discuss their results and not cite the literature (this was done in the introduction) - they should discuss what their results mean and what was the importance of looking at the stage, age and virus presence.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cancer biology sp breast cancer.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

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