

Retraction Notice

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History

Expression of Concern:

yes, date: yyyy-mm-dd

no

Correction:

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no

Comment:

This paper is retracted by the editor handling error.

This article has been retracted to straighten the academic record. In making this decision the Editorial Board follows [COPE's Retraction Guidelines](#). Aim is to promote the circulation of scientific research by offering an ideal research publication platform with due consideration of internationally accepted standards on publication ethics. The Editorial Board would like to extend its sincere apologies for any inconvenience this retraction may have caused.

Molecular Detection of Epstein Barr Virus in Women with Breast Cancer in the West Algeria

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Abstract

Breast cancer represents the most common malignant tumor afflicting women. This pathological condition remains the leading cause of death which constitutes an affliction that deserves considerable attention. As a result, the potential implication of viruses in its pathogenesis remains worth a hypothesis. The potential role of Epstein-Barr virus (EBV) in its pathogenesis is still a subject of continued discussion and investigations. The aim of this study is to evaluate a possible association between EBVs in breast cancers in western Algeria, and to determine the clinicopathological characteristics in order to specify the clinicopathological profile of tumors associated with this virus. We have searched the presence of EBV in 60 human breast cancer samples thanks to different techniques such as: PCR, *in situ* hybridization of EBER sequences and immunohistochemistry for latent membrane protein 1 (LMP1). The results obtained from this study showed the presence of this virus in only 16 cases or 26.6%. While the remaining 44 samples with a percentage of 73.3% showed a negative value. This may be due to sensitivity in the different techniques used and also what prompts us to suggest resuming the study using much more sensitive techniques such as real-time PCR. Our study indicates the presence of EBV DNA in a significant proportion of breast cancer in western Algeria. Further studies are required to clarify the role of this virus in breast carcinogenesis.

Keywords

Breast Cancer, Epstein-Barr Virus (EBV), Polymerase Chain Reaction (PCR),

1. Introduction

Cancerogenesis is a (multi-step) process characterized by complex cellular changes resulting in uncontrolled proliferation of malignant cells often developing from a normal cell. This development is caused by the accumulation of alterations in the genes responsible for the control of cell divisions and the maintenance of genomic integrity. The development of cancer is therefore, a multifactorial mechanism initiated by multiple agents such as the environment, genetics, irradiations and also the implication of viruses.

Indeed, during the past thirty years, several teams enabled to show the presence of different types of viruses which could play an important role in the genesis of many types of cancers in humans [1] [2].

Human viruses represent the second risk factor for the appearance (occurrence) of human cancers; the most common example is Epstein-Barr virus. This virus is now well-recognized as an oncogenic virus which is associated with a number of lymphomas as well as nasopharyngeal carcinomas and breast cancer [3]-[8].

The progression of EBV infection to malignant transformation involves two major phases: lytic and latent. Various EBV viral proteins are expressed during the two phases, which are interrelated. The latent proteins comprise six EBV nuclear antigens (EBNA1, 2, 3A, 3B, 3C, and LP), three latent membrane proteins (LMP1, 2A, and 2B), finally are also expressed two nuclear-localized noncoding RNAs: RNA EBER1 and 2 (EB encoded small RNAs) [9].

Based on EBV-related cancers, EBV latency has thus three classical types [9].

Latency I is observed in Burkitt's lymphoma and is identified by the expression of EBERs and EBNA1. Hodgkin's disease and NPCs are examples of latency Type II, wherein latent membrane proteins LMP1, LMP2A, LMP2B, nuclear antigen EBNA1, and EBERs are expressed. In latency Type III, all six EBNAs and the three EBV LMPs are expressed along with the EBERs. Latency Type III is observed in various lymphocytoid cell lines.

Despite recent progress carried out (undertook) in breast cancer, this tumor and heterogeneous disease remains a real major public health problem in western Algeria. Therefore, breast cancer constitutes the most common malignant tumor affliction and represents one of the main (leading) causes of death among women with an annual incidence rate of 20/100,000 women [10].

Although there are inherited genetic factors that influence the development of breast cancer and there are other factors increasing the risk for developing this cancer, its etiology remains largely unknown. Besides, virus infection can play a potential role in the same steps of the breast cancer pathogenic process.

This suggestion was based on the proven role of mouse mammary tumor virus (MMTV) as the causal agent of mammary tumors in mice [11]. Others have re-

ported that EBV might be associated with the pathogenesis of sporadic cases of human breast cancer [12], but this association is still a controversial topic. The aim of our current study is to evaluate the association between Epstein-Barr virus and breast cancer in West Algeria.

In this modest work, we tried to examine the presence of this virus in 60 tumor breast cancer samples using different techniques such as: Conventional PCR (Thermal cycler), EBERs *in situ* hybridization and immunohistochemistry for the latent membrane protein 1 (LMP1).

Furthermore, we investigated the relationship between the presence of this virus and several clinicopathological parameters in order to specify the clinicopathological profile of tumors associated with this virus (immunohistochemical expression of progesterone and estrogen receptors, and HER2).

2. Materials and Methods

2.1. Ethics

This study has received (obtained, get) the approbation (approval; agreement) from Ahmed Ben Bella University of Oran, in Algeria, and the Ethics Committee of the Regional Military University Hospital of Oran (RMUHO).

A written informed consent has been obtained from each patient after that the nature of the process has been completely explained.

2.2. Patients and Samples

In this study, we evaluated the prevalence of EBV human herpes virus in breast cancer in western Algeria using PCR (conventional on thermal cycler Fisher), immunohistochemistry and *in situ* hybridization techniques. We evidenced the presence of EBV genome DNA in 60 breast cancer cases from a forward study focusing on 130 female patients affected by breast cancer gathered together during these past three years, between 2010 and 2013 diagnosed at the anatomopathological laboratory of the Regional Military University Hospital of Oran (RMUHO).

The clinicopathological characteristics such as the immunohistochemical status of estrogen and progesterone receptors and HER2 expression of these patients were examined [13].

For all the patients, frozen tumor and matched adjacent normal breast tissue samples were available for viral investigation using PCR assays at LAPMO laboratory (Grenoble). Moreover, representative paraffin blocks from tumor tissues and matched adjacent normal breast tissues were chosen for viral analysis by immunohistochemical and *in situ* hybridization methods.

According to the WHO International Classification [14], the anatomopathological study reveals the presence of the following histopathological types:

Fifty-four samples were invasive ductal type.

Lobular histologic type was found in two cases, as to medullary histology type, our study evidenced the presence of three cases, and finally we found one case of inflammatory tumor type.

Data regarding patients' age, date of birth, menopausal status, tumor size, lymph

nodes and SBR (Scarff-Bloom-Richardson) histological grade [15] [16], were collected by the consultation of the anatomopathological reports and the re-reading of patients' clinical records.

This work has been realized in the anatomopathological laboratory of the Regional Military University Hospital of Oran (RMUHO) from an informed consent established between the different patients and their regular doctors. All the patients gave their approval for the use of their records and their tumor samples. Survival data were available for 39 patients.

2.3. Polymerase Chain Reaction (PCR)

DNA was extracted from frozen tumors and their corresponding normal breast tissues according to the standard extraction protocol.

For the detection of EBV, we have used the conventional PCR technique targeting the amplification of specific regions for the analyzed virus (EBV) [17]. The amplification is aimed at targets a 176-pb sequence located in the BamHI G region of the viral genome. A specific set of primers EBV-S (3'AACATGCTGTAT-GCCTCGCAGCG5') and EBV-R (3'AATTACTGGCGTGAATTGTGCCCA-5') was used for the amplification of a 176-pb sequence of gene in the BamHI G of the EBV genome [18].

The PCR reactions were performed using 400 ng of DNA extracted from each sample in a final volume and a total of 25 μ l, for each tube containing: 1 x PCR buffer; 200 μ M of each dNTPs; 0.2 μ M of each oligonucleotide primer and 1 U of Taq DNA polymerase (Promega).

Cycling conditions were as follows: Denaturation at 94°C for 3 minutes, followed by 40 cycles each of them comprising: a denaturation step at 94°C for 30 seconds; an annealing (hybridization) step at a specific temperature between 50°C and 60°C for each primer pair for 30 seconds and an extension (polymerase) step at 72°C for one minute and 30 seconds.

The reaction was finished with a 10-min extension at 72°C allowing Taq DNA polymerase to come to the end of synthesis of the polymerized fragments. The temperature and the duration of the cycle steps of specific amplifications for each primer pair are used for the detection of EBV by PCR.

For the detection of EBV, negative control sample containing the amplification mix without DNA as well as EBV positive control corresponding to DNA extracted from a tumor, this control (sample) came from DNA extracted from a patient's tumor affected by undifferentiated NPC (carcinoma of Nasopharyngeal), type (UCNT). Ten microliters of each amplified product were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide.

The sensitivity of PCR may lead to false positives because of contamination. For this reason, we used both standard PCR and *in situ* PCR techniques.

In situ PCR is less sensitive to contamination and has the important advantage of localizing the specific genetic material at the cellular level. Nevertheless, *in situ* PCR remains subject to both false positive and false negative results.

Consequently, we used stringent negative controls which comprised omitting

DNA primers and Taq polymerase.

2.4. *In Situ* PCR

Archival tissues on slides were washed in xylene to eliminate the wax followed by washes in decreasing concentrations of alcohol.

The tissues were subjected to pepsin digestion with varying times of digestion that was necessary for different tissues.

These differences were probably due to fixation procedures, which may vary in duration.

The digestion was stopped in 0.1 M Tris buffer pH 8.75 ml of PCR mix, containing inner nested PCR primers. Digoxigenin (DIG) – dUTP (0.3 nM) (Roche) was added to the tissue in a frame which was sealed.

PCR cycling was the same as standard PCR.

Detection using Anti-DIG-AP-Fab fragments (1 ml) (Roche) in buffer pH 7.5 followed by NBT/BCIP (2 ml) (Roche) in buffer pH 9.5 was stopped when a blue color was observed in the cells of the cancer specimen and not in the negative control. The tissues were counterstained with eosin. Any specimens that were positive in the negative controls showed that the DNA was self-priming and were unsuitable for *in situ* work (this is probably due to fragmented DNA acting as primers).

These samples were eliminated from the study. Any specimens that were negative for EBV virus were subjected to Beta-globin *in situ* PCR to confirm the result.

The electrophoretic pattern was photographed under ultraviolet light using the 2000 system (Bio-Rad, Marnes-La-Coquette, France).

Besides, extraction of DNA, PCR, gel electrophoresis were realized in the “Laboratory of Adaptation and Pathogenesis Microorganisms, LAPMO, UMR 5163 CNRS-Joseph Fourier University, Jean Roget institute; Grenoble”.

2.5. *In Situ* Hybridization

We used cold probes for the detection of Epstein-Barr virus RNA sequences of breast cancer. The detection of hybrids was realized by immunofluorescence (FISH). To evaluate the presence of EBV, we used the PNA (Peptide Nucleic Acid) probe (ref.: Y5200, DakoCytomation). This technique was realized in the pathological anatomy and cytology laboratory.

In situ hybridization was performed with EBER oligonucleotides, complementary to the two nuclear primary transcripts EBER 1 and 2 of the fluorescein isothiocyanate-conjugated EBV (Dako). Positive and negative controls were included in all series (each set). Positive controls were obtained by incubation with a sense probe and by incubation with antisense probe after digestion by RNase. The sections were deparaffinized, rehydrated then digested with proteinase K (1 µg/ml in TE buffer, pH 8), 30 minutes at room temperature. After incubation with the probe overnight at 42°C in a humid chamber, the sections were subjected to washes in TBS buffer pH 7.6.

Alkaline-phosphatase-conjugated antibody Anti-FITC was applied on the sections for 30 minutes. The sections were washed in two TBS pH 6 wash baths for 3 minutes. Then, BCIP/NBT chromogen was applied for 30 minutes.

After the revelation of marking and visualization in fluorescence using an Axio Cam MRm Zeiss microscope (Axioskop 2 plus HAL100-References: 98595; the system is managed by a microcomputer). The signal is located at the nucleus of tumor cell level. None of the nucleus of non-tumor cells marking was visualized. The specific green fluorescent nuclear localization signal is found in the majority of the nucleus of tumor cells.

All the silanized slides represent 50% positivity which were identified and expressed as dark brown granules at the nuclear level.

2.6. Immunohistochemistry

Immunohistochemistry technique allows analysis, localization and targeting of viral proteins (LMPs, EBNA, EBERS) in tumor cells by direct visualization, cellular components, tissue components, and deduction of potential functionalities.

However, this part of our study was realized in order to detect membrane protein LMP1 in our tumor specimens, using specific monoclonal antibodies oriented against this LMP1 protein (clones CS1-4, DakoCytomation) on 3 μ m thick histological sections.

After deparaffinage, unmasking and inhibition of endogenous peroxidase, we executed our technique by a 1/50 dilution of our antibody.

After the revelation of avidin-biotin-peroxidase complex, the slides were counterstained after application of 500 μ l of Harris' hematoxylin solution; finally, they were recovered by glass slides and mounted using resin (Pertex® Histolab Products AB, Gothenburg, SE).

The results obtained at this work showed a positive overexpression of protein LMP1 at the membrane and cytoplasmic level of our tumor cells.

2.7. Statistical Analysis

Fisher's chi-square χ^2 and exact test realized at the Laboratory "Modeling Environment and Prediction for Population Health" were used to look for an association between the presence of EBV, clinical and pathological parameters; the status of estrogen and progesterone receptors expression and HER2 over expression.

Overall survival was measured from the day of randomization until patients' death due to any cause *i.e.* 39/60.

All analyses were performed using SPSS software. For all of the tests, probability values of $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Detection of EBV

The presence of EBV virus was investigated on the breast tumor tissues collected from 60 women from western Algeria who were part of this study, as well as normal tissues coming from the same patients' population.

Figure 3. *In situ* hybridization for the detection of EBV-encoded small RNAs (EBERs). (Panel b): Breast carcinoma case showing an EBER negative signal in a normal lymphocyte infiltrating the tumor (tumor cells remain negative) (original magnification 400× and 1000×).

Figure 4. Cytoplasmic positivity of breast tumor cells for the detection the viral oncoprotein LMP1 of EBV by immunohistochemistry method (×400) (Panel c).

Figure 5. Cytoplasmic negativity of breast tumor cells for the detection of EBV LMP1 viral oncoprotein by immunohistochemistry method (×400) (Panel d).

3.3. Clinicopathological Characteristics of EBV-Positive Cases

Table 1 represents the comparative analysis results of the different characteristics of the clinicopathological and immunohistochemical data obtained in light of the different form of the EBV-positive and EBV-negative results from our breast tumors samples.

Overall, no correlation was found between the detection of EBV DNA by PCR and patient's age, histological grade, lymph node status, or tumor size.

HER2 expression by immunohistochemistry method did not vary significantly between EBV positive and EBV negative. With regard to hormonal receptors, EBV DNA presence correlates inversely with the expression of estrogen receptors ($P = 0.008$) (**Table 1**). Survival data were available for 39 patients with a median follow-up period of 40 months EBV positivity presents a trend toward a significant correlation with worse overall survival ($P = 0.193$) (**Figure 6**).

4. Discussion

Epstein-Barr virus (EBV) is a human herpesvirus type 4 (HHV-4) belonging to the Herpesviridae family. This family is made up of about fifty viruses, eight of which are strictly human. EBV was the first virus to be isolated from human tumors. Its

Table 1. Comparative analysis of clinicopathological features of positive-EBV and negative-EBV breast cancer cases.

Variable	Number of positive-EBV cases	Number of negative-EBV cases	Value of P ^a
Age (years)			
≤50	7	27	0.253
>50	8	18	
Histoprognostic grade			
Grade I	3	8	0.281
Grade II	5	16	
Grade III	8	14	
Tumor size (mm)			
≤20	3	10	0.415
>20	13	35	
Lymph nodes			
–	11	30	0.582
+	3	7	
Estrogen receptors			
–	12	21	0.008
+	4	23	
Progesterone receptors			
–	11	21	0.063
+	6	23	
HER2			
Absence of overexpression –	22	32	0.780
Presence of overexpression +	4	12	

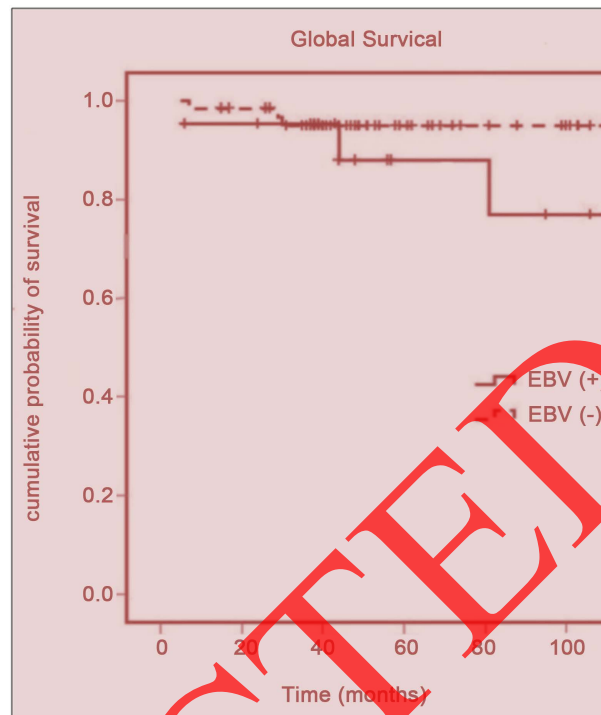


Figure 6. Distribution of EBV+ and EBV- according to clinical parameters. EBV(+) → N = 10; EBV(-) → N = 29; N = Patient.

implication in breast carcinomas was largely controversial. Indeed, its viral genome was reported by numerous authors in breast cancer with frequencies ranging from 20% to 66% [8] [12] [19]-[24].

In this study, we evaluated the prevalence of human herpesvirus (EBV) in women breast cancer from western Algeria using PCR, *in situ* PCR, *in situ* immunohistochemistry and hybridization techniques.

By comparing the results obtained at the end of our study with other authors' ones, we were able to identify the presence of EBV in a sampling (16/60) tested according to the previous different techniques. However, we observed that our results were compatible with the literature ones; it is possible that some prior findings may have been exaggerated by the phenomenon of lytic viral replication as identified by Huang *et al.* [25].

The identification of EBV in this current study, using PCR standard, *in situ* PCR and immunohistochemistry, is consistent with the findings of others authors.

False positive outcomes due to lytic viral replication are confined to standard PCR.

In this current study, we have shown by both *in situ* PCR and LMP 1 expression that EBV may be present in individual cancer cells. This observation is consistent with findings obtained by others [11] [12] [26] [27]. Consequently, our findings are likely to be accurate.

In this current study, analysis by PCR, demonstrate the presence of EBV DNA in 16 of 60 breast carcinoma cases *i.e.* (26.6%) analyzed. However, the remaining 44 samples showed a negative EBV. These outcomes could be due to the hetero-

geneity of tissues. The DNA of these samples was not collected from the zones infected by the virus. These data suggest that EBV is only found in tumor tissues.

We compared between our findings and other works performed by several teams, and our samples appear to be less carrier of the virus against the samples used by the other research teams and to the various studies reported in the literature.

Though it was not possible to definitely determine the identity of these cells, it may be that EBV positive lymphocytes have infiltrated breast tissues and transmitted EBV to breast epithelial cells.

PCR is potentially the most sensitive and the most specific method for the detection of viral genome DNA. But it does not allow the determination of the cellular source of any viral DNAs detected [28].

Indeed, according to the work of the researcher Preclado, he showed and, it is argued that EBV infected lymphocytes are in the tumor stroma, which might explain the detection of EBV in breast tumor tissue by PCR [29]. In the same research context, studies by Chu and colleagues have shown that the localization of the viral genome at the cellular level is only possible by the application of morphological techniques as *in situ* hybridization or immunohistochemistry [30].

However, according to Labrecque and his colleagues, LMP1 oncoprotein was detected by immunohistochemistry method whereas Lespagnard and his colleagues have detected in 1995 this same protein in invasive breast carcinomas using conventional PCR.

Immunohistochemistry targeting viral oncoprotein LMP1 expression is a widely-employed assay that is sensitive but limited by the fact that LMP1 is absent in some otherwise EBV-related tumors.

The very limited detection of LMP1 protein in our breast tumor samples is consistent with the lack of expressions reported in non-Hodgkin's lymphomas or cancers types [31].

The influence of this virus may be reflected in the differences in breast cancer morphology and phenotypes.

In agreement with our results, several immunohistochemical studies targeting LMP1 protein expression in breast cancer had negative results [21] [22] [26] [30] [32]. Also, in the same context, we looked for EBERs in our tumor samples knowing that the latter are highly abundant in the EBV-infected cells at a rate of 10^5 to 10^7 copies per cell [33], so we have used *in situ* hybridization technique.

The results obtained from this experimental part show that the majority of our samples are negative for EBERs.

Therefore, it is possible that EBERs are not expressed in breast tumor cells or that they are expressed in low quantities, indicating that breast cancer is unlike traditional EBV-related tumors, which strongly express EBERs in virtually all tumor cells [12]. But this hypothesis remains to be proven and it is contested by several authors.

This result allows us to deduct that viral DNA detected by PCR in breast cancer cases analyzed could originate from lymphocytes infected by EBV and infil-

trating tumor stroma. Among all the previous studies regarding the evaluation of EBV presence in breast cancer by *in situ* hybridization [12] [22] [26] [27] [28] [31] [32] [34] [35] [36], positive results were found in only three [22] [34] [31].

In these three studies, the detection of RNA EBERS only comprised a small proportion of tumor cells [34].

Besides, Lin *et al.* [7] [15] [37] have reported that the different types of breast carcinoma cells infected by EBV lead to activation of the HER2/HER3 signaling axis in these breast cancer cells. These data are consistent with other previous findings indicating overexpression of HER2 [38], which suggest that EBV infection may impede clinical chemotherapy in breast cancers. In our current study, we found no correlation between EBV presence and overexpression of HER2. Conversely, we observed a significant correlation between the presence of EBV and the negativity of estrogen receptors ($P = 0.008$). Moreover, the analysis of survival data of our patients, according to the status of EBV, indicated a difference in the survival rate between patients, but this difference did not reach the threshold of statistical significance. These results suggest an aggressive phenotype of EBV-positive breast carcinoma cases.

Our results are in accordance with some previous studies that found a stronger EBV association with features of more aggressive breast tumor such as tumor size, SBR grade, number of positive lymph nodes (>3), and negativity of hormone receptors [12] [30] [34].

The non-significant trend that the presence of EBV is associated with higher grade breast cancer is consistent with the recent observations by Mazouni *et al.* [39].

By reviewing literature data, we did not find published studies which tried to investigate the prognostic impact of EBV presence in breast cancer.

Other studies including a more important number of cases are necessary to evaluate the prognostic significance of EBV presence in breast cancer.

There were no obvious associations between the presence of virus EBV in breast cancer and expression of ER, PR, HER. The number of cases is too small to make confident implications.

EBV has been identified in a range of body organs but with greatly varying viral loads [8].

Consequently, this virus may be cell type specific and not organ specific.

EBV has been associated with Hodgkin's lymphoma, Burkitt's lymphoma and nasopharyngeal cancer.

5. Conclusions

We have detected by PCR, the presence of DNA sequences belonging to EBV viral genome in 26% of breast cancer cases in western Algeria. Further studies are necessary to clarify the role of this virus in breast carcinogenesis. The absence of this virus at the tumor cell level suggests that it is not implicated in breast cancer pathogeny in our population of study.

We conclude that the association of Epstein-Barr virus with breast carcinomas

remains a very controversial idea, indeed; the following authors Perrigoue and his collaborators in 2005 [40] as well as Perkins *et al.* in 2006 [41] have reported negative results for the link between EBV and breast cancer, so we can associate this divergence of results with the fact that samples used by several teams are either fixed or fresh. Therefore, the tissue used during the experiment could play a key role in the presence of this virus. We can deduct that EBV has an oncogenic role in human breast cancer.

The presence of this virus in breast cancer is associated with young age of diagnosis and possibly an increase grade of breast cancer and EBV may take part in some breast cancers.

Breast cancer thus remains an extremely heterogeneous and multifactorial pathology.

However, it seems to be imperative to spread our study on a more important sample and to use other techniques such as real-time PCR, sequencing and DNA chip.

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