

# Incidence of Epstein–Barr virus in Syrian women with breast cancer: A tissue microarray study

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**Keywords:** breast cancer, Epstein–Barr virus, invasive cancer, Syrian women

Epstein–Barr virus (EBV) has been recently shown to be present in human breast cancer worldwide, which could play an important role in the initiation and progression of this cancer. In this regard, we aimed to explore the prevalence of EBV in 108 breast cancer tissues from Syrian women using polymerase chain reaction (PCR) and tissue microarray (TMA) analysis. We found that EBV is present in 56 (51.85%) of breast cancer samples. Additionally, we report that the expression of LMP1 gene of EBV is associated with a cancer invasive phenotype in the majority of the cancer samples. These data imply that EBV is present in breast cancer worldwide including Syria and its presence is associated with more aggressive cancer phenotype. Thus, future investigations are needed to elucidate the exact role of EBV in breast carcinogenesis and metastasis.

## Introduction

Epstein–Barr virus (EBV) is a ubiquitous gamma-herpesvirus that infects more than 90% of the population worldwide.<sup>1</sup> Besides infectious mononucleosis EBV is well known to be linked to several human cancers, including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinomas (NPC), gastric cancer and T-cell/natural killer cell lymphoma.<sup>1,2</sup>

EBV-infected cells express a small subset of genes, including 6 nuclear antigens (EBNA-1, -2, -3A, -3B, -3C, and -LP), 3 latent membrane proteins (LMP-1, -2A, and -2B), 2 small noncoding RNAs (EBER-1 and 2), and BamHI-A rightward transcripts (BART).<sup>3</sup> The differential expression pattern of these latent genes defines the distinct latency programs associated with the types of cancers.<sup>2,4</sup> For instance, type II latency is characterized by a more restricted latent gene expression pattern (EBNA-1, LMP-1, -2), and is associated with Hodgkin's lymphoma and NPC as well as other carcinomas including gastric and probably breast.<sup>4,5</sup> Thus, LMP1 is considered the major EBV-encoded oncogenic protein, as it induces a multitude of effects promoting cell growth, protecting cells from apoptosis, enhancing cell motility, promoting angiogenesis and it is frequently expressed in EBV-associated human NPC.<sup>6,7</sup> On the other hand, EBNA2 is considered the central transcriptional regulator of viral and cellular genes that are involved in B cell immortalization.<sup>8</sup> LMP2A plays a key role in regulating viral latency and it functionally mimicks signals induced by the B cell receptor, altering normal B cell development; LMP2A can also immortalize epithelial cells and influence their adhesion and motility.<sup>6,9</sup> Alternatively, EBV is the first, and

one of the few viruses known to encode micro-RNAs (EBV-miRNAs).<sup>9</sup> Accumulating evidence suggests that miRNAs play important roles in carcinogenesis, modulating apoptosis and host innate defense mechanisms.<sup>9,10</sup> All this implicates EBV in the initiation and progression of human malignancies including several types of lymphoma and carcinomas.

Several recent studies reported that approximately 30–50% of human breast cancers, worldwide, are positive for EBV;<sup>11–15</sup> controversially a few studies revealed that EBV could not be detected in human breast cancers.<sup>16,17</sup> Nevertheless, earlier studies pointed out that the presence of EBV in human breast cancers is associated with more aggressive features and poor prognosis.<sup>15,18</sup> Thus, these studies suggest that EBV could play an important role in the progression of breast cancer.

This study aims to investigate the presence of EBV and its association with tumor aggressiveness in breast cancer in Syrian women.

## Results

In order to determine the incidence and the role of EBV infections in human breast carcinogenesis in Middle Eastern women, we investigated the presence of this virus in a cohort of 108 breast cancer samples from Syrian women by PCR and Immunohistochemistry (IHC) analysis using specific primers for LMP1 and/or EBNA1 genes of EBV (Table 1) and a monoclonal antibody for LMP1 (please refer to Materials and methods section and below). Our study revealed that 56 (51.85%) of the 108

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Submitted: 07/28/2014; Revised: 10/23/2014; Accepted: 11/07/2014

<http://dx.doi.org/10.1080/21645515.2015.1009342>

**Table 1.** The specific primer sets for LMP1 and EBNA1 genes of EBV used for PCR amplification

Genes	Primers
LMP1	5'- TTGGAGATTCTCTGGCGACT -3' 5'- AGTCATCGTGGTGGTGTTC A -3'
EBNA1-297	5'- AAGGAGGGTGGTTTGAAAG -3' 5'- AGACAATGGACTCCCTTAGC -3'
EBNA1-207	5'- ATCGTGGTCAAGGAGGTCC -3' 5'- ACTCAATGGTGAAGACGAC -3'
GAPDH	5'- GAAGGC-CATGCCAGTGAGCT -3' 5'- CCGGGAAACTGTGGCGTGAT -3'

samples are EBV positive; in contrast, 52 (48.15%) cancer tissues were negative for EBV (Table 2A).

On the other hand and to assess the association between the presence of EBV and tumor aggressiveness in breast cancer in Syrian women, we examined the expression of the LMP1 oncoprotein of EBV and its association with invasive cancer phenotype in our breast tissue samples by H&E and IHC using tissue microarray methodology. We found that the LMP1 expression, a cytoplasmic dot staining pattern, is correlated with invasive breast cancer phenotype in 60.7% of breast cancer samples as opposed to 20.8% of *in-situ* cancer tissues (Table 2B and Fig. 1); while we presume that these *in situ* breast carcinomas, which are EBV-positive, will ultimately progress into invasive carcinomas under the effect of EBV, since they are already intermediate to high

**Table 2A.** EBV detection in breast carcinomas by PCR. The incidence of this virus was found in 58 samples out of 108 examined using specific primers for LMP1 and EBNA1 genes of EBV

	Tested cases	Positive	Percentage
Breast cancer tissues	108	56	51.85

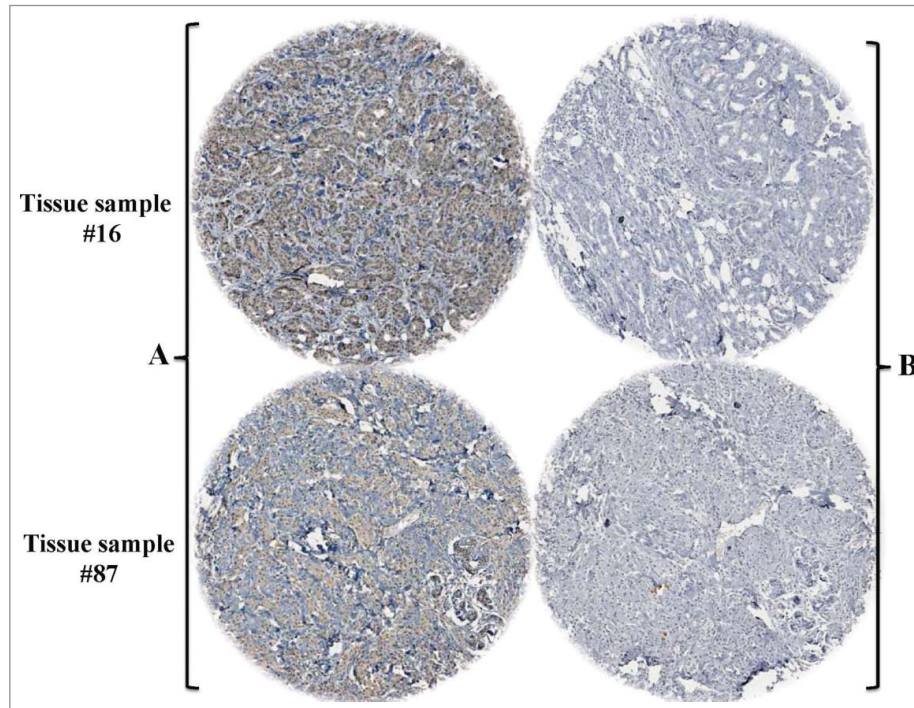
**Table 2B.** Presence of EBV and its association with invasive breast cancer phenotype. The presence of EBV is more frequent in invasive breast cancer in comparison to *In Situ* breast cancer tissues ( $P = 0.0013$ ); the presence of EBV was assessed by PCR for LMP1 and EBNA1 genes and confirmed by IHC analysis of LMP1 expression using Tissue Microarray (TMA) methodology, as described in the Materials and Methods section.

Breast cancer	No of cases	EBV+(%)
Invasive	84	51/84 (60.7)
<i>In situ</i>	24	5/24 (20.8)

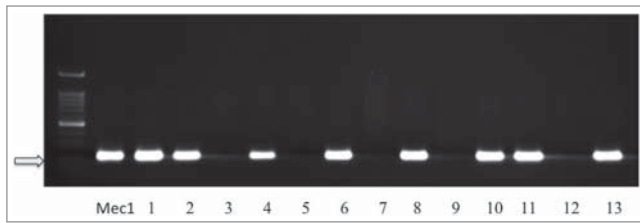
nuclear grade. On the other hand, we noted that more than 90% of the cancer cells in our samples were positive for LMP1 in comparison with the adjacent normal mammary epithelial cells, which were revealed negative for LMP1 (Fig. 1). The IHC data were confirmed by the presence of both LMP1 and EBNA1 in 54 of our samples using PCR analysis with all the necessary controls including Mec1 and MCF7 cell lines as well as normal mammary epithelial cells (Fig. 2). Accordingly, we were able to prove that EBV is present in the majority of invasive breast carcinomas in comparison with *in situ* cancers in Syrian women (Table 2B and Figs. 1 and 2). Thus, the presence of EBV in breast cancer could play an important role in its progression.

## Discussion

This is, to the best of our knowledge, the first study on the presence of EBV and its relation with tumor aggressiveness in breast cancer in Syrian women. In fact, recently there has been an increasing interest in investigating the possible role of viral infection in breast cancer especially EBV and human papillomaviruses (HPVs) based on their roles in various types of cancers such as lymphoma and cervical, respectively. Accordingly, we have recently reported that 61% of human breast cancers in the Syrian population are positive for high-risk HPVs.<sup>19</sup> In addition, our study revealed that the presence of high-risk HPVs is correlated with an invasive and metastatic breast cancer form. Regarding the presence of EBV in breast cancers worldwide, several recent investigations reported that approximately 30–50% of these cancers



**Figure 1.** Representative IHC revealing LMP1 expression of EBV in 2 invasive breast cancer tissue samples (A): with LMP1 antibody; (B): without antibody, control). Magnification is 100X. This analysis was performed using TMA methodology with all the necessary controls; and presence of EBV was confirmed by PCR using specific primers for LMP1 and EBNA1 genes in 54 cases of our samples including these samples.



**Figure 2.** Representative PCR reactions for LMP1 of EBV in 13 different breast cancer tissue samples. LMP1 and EBNA1 are present in 8 of 13 samples (line 1: Mec1 cell line was used as a positive control; MCF7 and normal mammary epithelial cells were used as negative controls, data not shown).

are positive for EBV;<sup>11-15</sup> however, a small number of studies were unable to detect EBV in breast cancer.<sup>16,17</sup> In parallel, it is important to emphasize that there are only few studies about the presence of EBV in the Middle East which can be outlined as follows: Kalkan et al.,<sup>20</sup> revealed that 58% of human breast cancer and normal mammary tissues are positive for EBV in the Turkish population. Zekri et al.<sup>21</sup> found that, 45% and 28% of breast cancer in Egyptian and Iraqi women, respectively, are positive for EBV. In parallel, Hachana et al.<sup>22</sup> demonstrated that 27% of breast cancer tissues in Tunisia are positive for EBV. However, a recent study from Iran showed that EBV is not detectable in breast cancer and normal mammary tissues in Iranian women.<sup>17</sup> Therefore, investigations from different countries of the Middle East area, except one study from one country, showed that the presence of EBV in breast cancer and mammary tissues vary from 27 to 58%, which is approximately similar to its presence in breast cancer worldwide. In this study, we report that EBV is present in approximately 52% of breast cancer in Syrian women. Therefore, our data point-out that the prevalence of EBV in breast cancer tissues in Syria is similar to the incidence of this virus in women worldwide including the Middle East. On the other hand and based on our previous HPV's investigations in human breast cancer and the present study, as well as our recent paper about HPV and EBV cooperation in human oral cancer,<sup>19,23</sup> we believe that high-risk HPV's could cooperate with EBV in human breast carcinogenesis and metastasis; this presumption is presently under-investigation. In this context, it is important to mention that ~20% of our human breast cancer samples revealed positive for both HPV and EBV together.

Regarding the association between EBV and tumor aggressiveness, recent studies have reported that the presence of EBV is correlated with human invasive carcinomas including breast and nasopharyngeal.<sup>11,18,24</sup> Moreover, earlier investigations demonstrated that LMP1 and EBNA1 onco-proteins of EBV enhance cancer progression and metastasis of nasopharyngeal malignancy through the initiation of the epithelial-mesenchymal transition (EMT) phenomenon,<sup>24-26</sup> which is a crucial event during cancer metastasis progression.<sup>27</sup> Therefore and based on this study and others, we believe that the presence of EBV in breast cancer could enhance cancer progression through the initiation of EMT event via EGF-receptor and/or Akt signaling pathways, as previously described in gastric and nasopharyngeal carcinomas;<sup>9,24</sup> in

addition, we assume that Wnt/ $\beta$ -catenin signaling can be involved in this progression since it has been demonstrated that LMP1 of EBV regulate this pathway in nasopharyngeal carcinomas.<sup>28</sup> These signaling pathways can deregulate the expression patterns of several key genes of cell adhesion and invasion such as E-cadherin, Fascin as well as others and consequently enhance breast cancer progression (this hypothesis is presently under investigation in our laboratory).

In conclusion, we demonstrate that EBV is present in human breast cancer in Syrian women. In addition, EBV infection was found in approximately 52% in breast cancer, which is similar to its presence in breast cancer in Middle Eastern women, based on Kalkan et al.<sup>20</sup>, Zekri et al.<sup>21</sup> and Hachana et al.<sup>22</sup> studies. In parallel, we report that the presence of EBV in breast cancer is associated with more aggressive phenotype. Our findings provide clear evidence on the presence of EBV in human breast cancer and its relation to the progression of this cancer. However, we believe that further studies are required to elucidate the role and pathogenesis of EBV in human breast cancer, especially since EBV vaccine is presently under clinical trial investigations. Therefore, it is of great interest to gain a better understanding of the association between EBV infection and breast cancer initiation and progression.

## Materials and Methods

### EBV detection and PCR analysis

A total of 108 blocks from breast cancer patients with a median age of 52 (range 26–66) years were used in this study. Formalin fixed (buffered neutral aqueous 10% solution), paraffin embedded tumor materials were obtained from the department of Pathology/Faculty of Medicine, University of Aleppo. The use of these specimens and data in research was approved by the Ethics Committee of the Faculty of Medicine of Aleppo University.<sup>19</sup> Five  $\mu$ g of purified DNA (from each sample) was analyzed for EBV by PCR using specific primers for LMP1 (Table 1) and EBNA1 genes as described by Glenn et al.<sup>11</sup> Briefly, LMP1 gene was amplified for 35 cycles of 95°C for 30s, 60°C for 30s, and 72°C for 30s. In parallel, EBNA1 was amplified in 2 rounds; the first one produced a 297 bp fragment using EBNA1–297 primers. The product of the first round was used as a template for the second amplification using EBNA1–207 primers, generating a 209 bp fragment. The amplification condition of the EBNA1 was 35 cycles of 95°C for 30s, 55°C for 30s, and 72°C for 30s. DNA from the chronic B cell leukemia, Mec1, and MCF7 cell lines as well as specific primers for the GAPDH gene were used as controls for the presence of EBV genes and PCR analysis, respectively, (Table 1). On the other hand, DNAs from human normal mammary epithelial cells were used as negative controls for our PCR analysis.

### Tissue microarray

The tissue microarray (TMA) construction was performed as described previously by our group.<sup>19</sup> Briefly, tissue cylinders with a diameter of 0.6 mm were punched from representative tumor



areas of a 'donor' tissue block using a semiautomatic robotic precision instrument. Two sections of the TMA blocks were transferred to an adhesive coated slide system (Instrumedics Inc., Hackensack, New Jersey). Slides of the finished blocks were used for H&E and immunohistochemistry analysis.

### Immunohistochemistry (IHC) analysis

Immunohistochemical procedures examining the expression of LMP1 was carried out using standard procedures as previously described.<sup>19</sup> Primary LMP1 antibody was obtained from Dako. Briefly, TMA sections were deparaffinised, rehydrated and endogenous peroxidase activity within the rehydrated tissue was blocked with a solution of 3% hydrogen peroxide in methanol for 10 min at room temperature. The TMA slides were cooled and equilibrated in Optimax<sup>TM</sup> wash buffer then incubated overnight (15 h) at 4°C with the LMP1 primary antibody (mouse monoclonal; clone 1-4; Dako, Canada). Sections were then washed (4x1 min), and the appropriate secondary HRP-conjugated antibody was applied for 1 h at room temperature (Calbiochem, Canada). The slides were counterstained with haematoxylin and mounted. Finally, sections were analyzed by Leica DMLB microscopy equipped with a Leica DFC-480 Camera (Leica Microsystems, Germany). Immuno-satining analysis

on human normal mammary epithelial cells were used as control for LMP1 expression using the same antibodies.

### Statistical analysis

Statistical analysis was performed using SPSS (version 22) and R. The data were assessed as nonparametric data. We used  $\chi^2$  test with Yates correction to assess the significance of the relationship between tumors aggressiveness and EBV presence.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Acknowledgments

We are thankful to Ms A. Kassab and Mr. B. Al Moustafa for their reading of the manuscript. We also thank Dr. N. Benlilame for her technical assistance.

### Funding

This work is supported by the Canadian Institutes for Health Research and the Fonds de la Recherche en Santé du Québec (FRSQ- Réseau du Cancer).

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