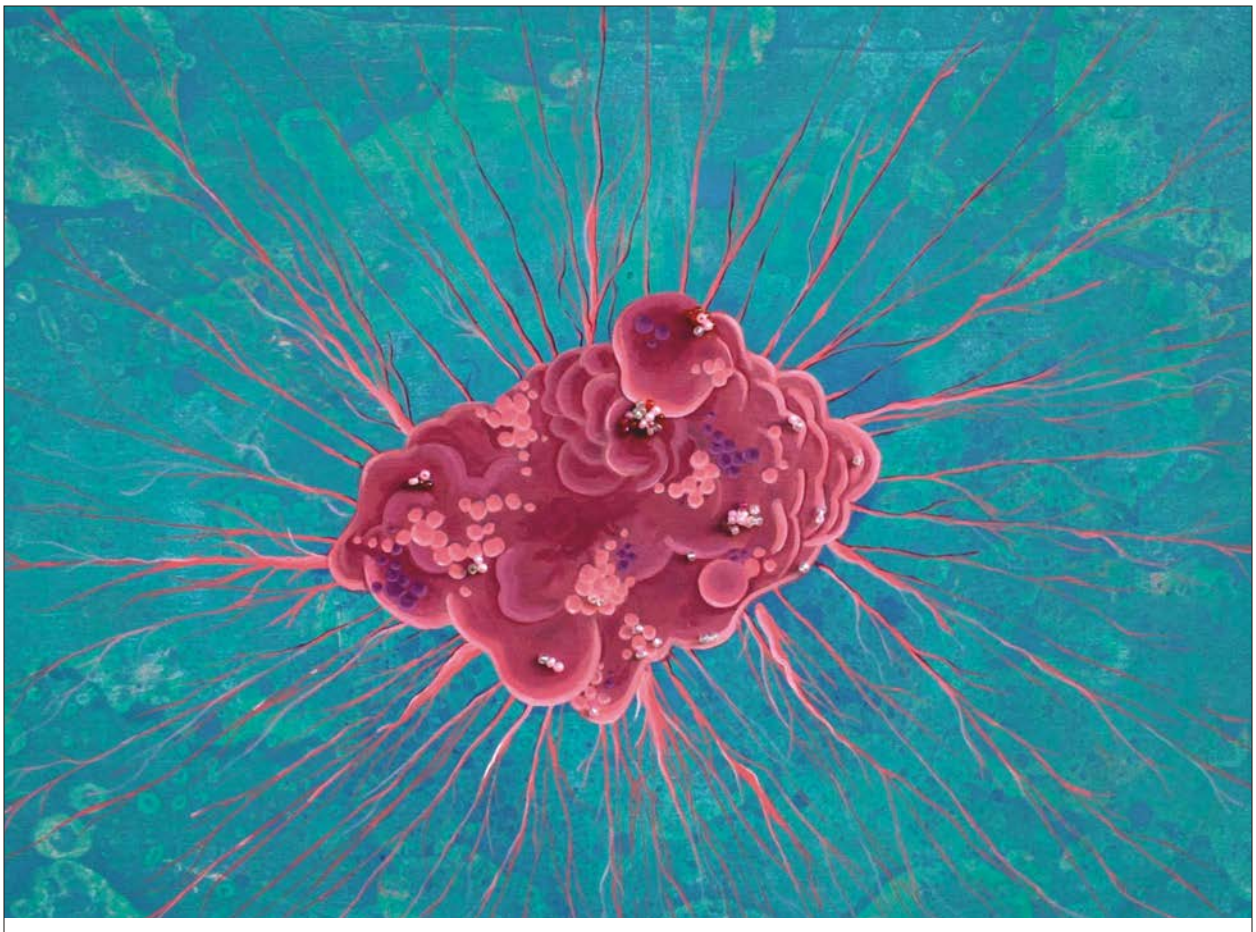

International Journal of Cancer Research

ISSN 1811-9727

Academic Journals Inc.

www.academicjournals.com

International Journal of Cancer Research 11 (1): 32-40, 2015
ISSN 1811-9727 / DOI: 10.3923/ijcr.2015.32.40

Research of Epstein-Barr Virus Genome and Quantification of Viral Load in Algerian Frozen Tissue of Breast Cancer

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ABSTRACT

Nearly all persons are infected with Epstein-Barr Virus (EBV) and remain infected all their life. While almost all EBV infections are benign a small percentage of infected persons, develop certain cancers. EBV is associated with 100% of the undifferentiated NPC (nasopharyngeal carcinoma). This type of cancer is increasing in Algeria (endemic area of EBV) and that other forms of cancer are supposed to be linked to EBV in this region. Recent studies suggest a link between EBV and breast cancer which can provide new knowledge and help to identify women at risk, using the virus as a tumor marker. However the association of EBV with breast cancer remains controversial. In this study, an investigation on the presence of EBV by quantifying viral load in frozen biopsies breast tumors among women in western Algeria, by real-time PCR (Q-PCR). A study on the presence of EBV and the quantification of the viral load in breast tumors (frozen biopsies) from western Algerian has been made by using quantitative real time PCR (Q-PCR). These results show that the EBV genome was detected in approximately 78% of tumor samples and in different DNAs extracted in several pieces within the same tumor; however the number of copies of EBV remains low. The viral load was found to be highly variable from one tumor to another and within the same tumor and DNA extracted from the same sample was positive in one case and negative in others. EBV genome is heterogeneously distributed in the tumor and with a threshold of low positivity and negative results could be due either to its poor storage of samples or to the heterogeneity of cancerous tissues or even at the limit of sensitivity of the technique used.

Key words: Breast cancer, EBV, viral load, Q-PCR real time, diagnostic, Algeria

INTRODUCTION

Breast cancer is the most common cancer affecting women in developed countries (Key *et al*., 2001). In Maghreb, breast cancer data are different from those of Europe, incidence of this cancer is lower, average age of diagnosis is younger and the size and the stage of tumors is higher. In Algeria the breast cancer is the first cause of mortality among women; the number of breast cancer cases has significantly increased during the last years, with rough rate of 29.8 and standardized rate of 37.1 per 100.000 populations. There are several factors that can increase the risk of breast cancer; however genetic predisposition was found to be one of the important risk factors (Ford *et al*., 1998). Breast cancer remains a heterogeneous disease; its aetiology is not completely understood; yet, a possible viral aetiology for breast cancer has been proposed and Epstein-Barr virus is an important research candidate (Joshi *et al*., 2009). EBV might play a role in breast cancer development or progression (Bonnet *et al*., 1999; Arbach and Joab, 2005). The EBV, a ubiquitous

human herpes virus, is associated with an increasing number of lymphoid and epithelial malignancies (Rickinson and Kieff, 1996). Several authors have reported detection of EBV in a subset of breast carcinomas (Labrecque *et al*., 1995; Luqmani and Shousha, 1995; Fina *et al*., 2001; Arbach *et al*., 2006). EBV has two distinct life cycles in the human host; a lytic form of infection and a latent form of infection that allows the virus to persist in a dormant condition for lifetime in the host. During the latent form, the genome remains in an episomal status and the expression is restricted to three latent membrane proteins, six EBV nuclear antigens and two small RNAs which are non-polyadenylated (Yin *et al*., 2004). Fina *et al*. (2001) showed a positive ratio of 40% (16/40) of each inflammatory and non-inflammatory Algerian samples. In 2006, EBV genome has been detected by Murray (2006) in 10% of samples using Q-PCR.

Bonnet *et al*. (1999) have reported the presence of EBV genome in 51% of breast carcinomacases. In 2006, Arbach *et al*. (2006) indicated that the viral load found in breast cancer is low, this group have also performed quantification of EBV DNA in microdissection of different tumours and observed a large heterogeneity in distribution of viral genomes from one region to another within the same tumour, as well as among different ones. Negative results have also been reported (Hermann and Niedobitek, 2003; Lespagnard *et al*., 1995). Studies concerning the distribution and frequency of the viral load EBV in different geographical regions were conducted using the semi quantitative PCR and quantitative real-time PCR by comparing regions of high NPC risk with those of low risk.

Geographic and ethnical factor might influence the role of EBV in the pathogenesis of breast cancer (Lopategui *et al*., 1994).

In order to give an answer on possible geographical influences on the frequency of the genome load of EBV and breast cancer, this study has been undertaken using quantitative real time PCR to detect EBV genome and to quantify the copy numbers of viral load in tumours from Algerian breast cancers.

MATERIALS AND METHODS

Patients and characteristics of the selected group: The study was conducted on 64 patients; all of them were women and born in west of Algeria (Oran), the tumours were provided by Central University Hospital of Oran from Chirurgical and Gynaecological Centre, the median age of the patients was 46.34 years (24-79 years). Among 64 patients, 20 had at least one case of breast cancer in their family. Biopsy specimens of breast carcinoma were collected between July 2005 and January 2006; tumour specimens were obtained with the agreement of the patients, according to the protocol (for the use of surgical tissues and medical records) previously approved by the local human studies committee. Patients were informed about the subsequent study procedures and informed consent was sought.

The sample was divided into two parts; the first part was used for histological characterization and histoprognostic grading (SBR: Scarff-Bloom and Richardson classification) according to the method of Contesso *et al*. (1987) and Singletary *et al*. (2002) and the second part was immediately frozen and stored at -80°C.

All histopathological slides were re-analysed independently by two trained pathologists and a consensus diagnosis was obtained. All cases of malignancy, size of the tumour, histological type and grade of the tumour, were noted.

DNA extraction and PCR amplification: DNA from frozen samples was extracted using Quiamp DNA mini-kit (QIAGEN, Courtaboeuf, France) according to the manufacturer's instructions, the extraction was carried out in three different areas of the same tumor and real time Q-PCR was applied to quantify the copy number of EBV genome from samples. Q-PCR of the EBV thymidine kinase gene (BXLF-1)169 pb was performed as described by Brengel-Pesce *et al.* (2002). Dilutions of DNA extracted from Namalwa cells, containing two integrated copies of EBV genome per cell (Lawrence *et al.*, 1988), were used as an external standard. Amplification of the EBV genome was performed using a number of cycles ranging from 25 to 39. Total genomic DNA was quantified by amplification of the β-globin gene (human genomic DNA) with Roche kit (Control kit DNA). Standardization was performed with DNA from the Light Cycler control kit (Roche Molecular Diagnostic Meylan, France). The calibration curve allowed the establishment of the following equation for the number of EBV genome: y = -3.33x+39.5, with an R2 value of 1 and an error of 0.0941. The kit Roche (LC Fast Start DNA Master Hybridization probes) using a curve of DNA of Namalwa, tumor sample DG75 (EBV negative) and H2O. The protocols used are the same as cited by Arbach *et al.* (2006).

RESULTS

Histological study: Tumors type were mainly infiltrating ductal carcinoma, 54.68% of the cases were infiltrate ductal carcinoma grade II and III (SBR) (Fig. 1); lobular infiltrate carcinoma was found in 15.62% of cases (Fig. 2), 6.25% of the cases were polymorph lobular carcinoma, colloid mucous and tumor phyllode (Fig. 3) and other types in 23.46%. The tumor size varied from 1-4 cm in diameter.

Viral load in breast cancer tissue specimens: Q-PCR assays were performed on all samples of breast cancer biopsy tissues. In 14 cases, no copies of the EBV genome were amplified or the number of copies was below the threshold of detection (Fig. 4).

Fifty samples (78, 12%) were considered positive for EBV since 0.002 to 109 copies were detected in the assay. Table 1, shows the number of copies of the EBV genome detected in each of the 50 EBV-positive tumor samples.

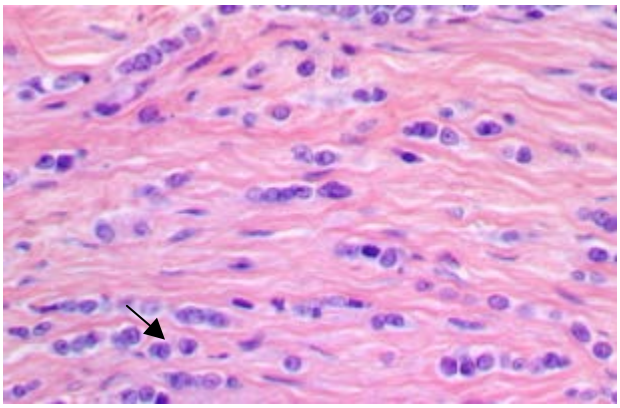

Fig. 1: Infiltrating ductal carcinoma

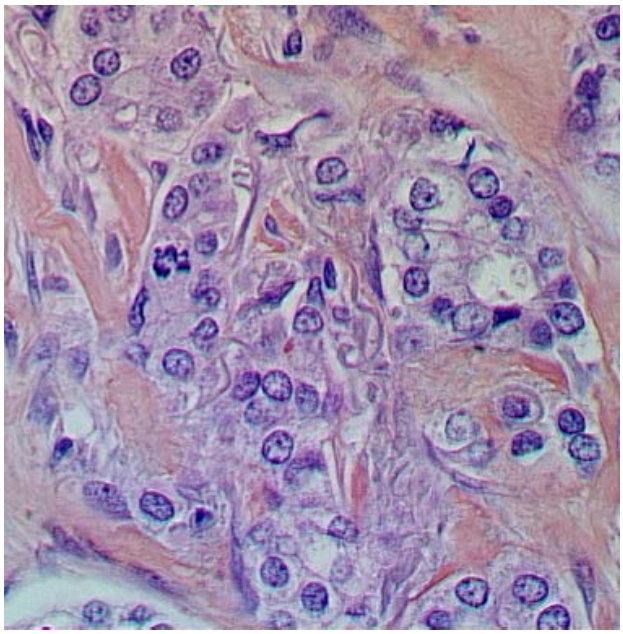

Fig. 2: Invasive lobular carcinoma

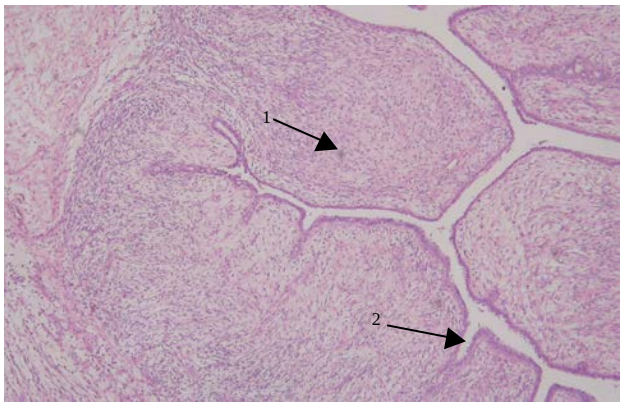

Fig. 3: Phyllodes tumour, (1) Contingent mesenchymal: Stroma abundant infiltrating the adjacent breast parenchyma and (2) Epithelialquota: Ductallining made of adouble nontumor typical cellbase (internal and external gland myoepithelial)

The copy number per 1,000 total cells is determined assuming that one cell contains 6 pg of DNA. The number of EBV genomes was very low. A measurement was done in 24 samples; 0.002 to 0.09 copy per 1,000 total cells and in 20 samples; 0.09 to 1 copies per 1,000 cells. In 4 samples; 2 to 24 copies per 1,000 cells. In one sample, exhibiting the highest load; 109 copies per 1,000 cells were measured. In different analyzed DNA, from the same tumors we have detected a very heterogeneous distribution of the viral load (Fig. 5). We have extracted from patient A1, DNA from the same tumor in 5 different places, this sample shown a heterogeneous repartition of viral load.

Table 1: No. of copies of EBV genome

Samples[a]	Copies/100 cells[b]
A1	109-3-10-210
F96	0-0-0-24-0.012
C43	0-15-0.08
G87	0-3-4
X15	2-0-0.016
X3	1-0.16-0.020
J10	0.08-0.31-0.003
C23	0.70-0.05-0
H8, H18, H38	0-0.70-0-0
G57	0-0.70-0.17
F36	0.62-0.12-0.07
G77	0-0.08-0.5
A91	0-0.34-0.009
X6	0-0.28-0.10
E55	0.2-0.026-0
F46	0-0.24-0.05
AS1	0.04-0-0.25
C53	0-0.08-0.18
H38	0.17-0-0.017
D84	0.10-0.13-0
A61, A71	0.03-0.12-0-0.060.07
X9	0-0.10-0
H28, H48	0-0.12-0.016
X5	0-0-0.10
D64, D74	0.10-0.03-0.05
G67, D94, D94	0-0.09-0.05
G27	0.04-0.034-0.08
X14	0-0.07-0
X7	0.010-0.079-0.026
B32	0.05-0-0
F6	0.045-0-0
B52	0.042-0
G37	0-0.04-0.027-0
C33	0-0.04-0
C13	0.04-0.03
X1	0.043-0-0
l9	0.034-0.020
D4	0.03-0.02-0-0.03-0
J80	0-0-0.03
D34, D44, D54	0-0-0.02
E5	0.02-0-0
J50	0-0-0-0.022
X16	0.022-0-0
J100	0-0-0.014
B82, B92	0-0-0.01
J40	0-0.014-0
X2	0-0.012-0
F26	0-0.007-0
B72	0-0.003-0
F76	0.002-0-0

[a]Corresponding to samples of breast cancer from A1 to X16, [b]Corresponding to the distribution of the No. of copies per 1,000 total cells

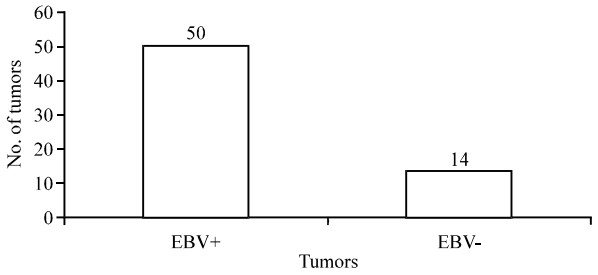

Fig. 4: Distribution of tumors EBV positive and EBV negative

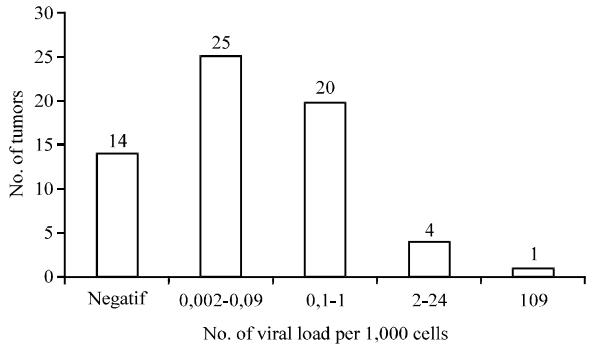

Fig. 5: Viral load from different categories

This observation was also available for other DNA samples. As the patient F96 for example shows significant variations in viral load, in the three extracted DNA, in the first amplified DNA we calculated 24 copies per 1.000 cells, 0.012 in the second DNA and no copies were observed in the last amplified one.

These finding shows a very high heterogeneity in viral load distribution, within the same tumors at different locations.

DISCUSSION

Detection of EBV in breast cancer has generated a great deal of controversy; some authors suggested that positivity with EBV is most likely caused by the presence of some infected lymphocytes in the tumors samples (Perrigoue *et al.*, 2005; Xue *et al.*, 2003).

Although, a part of the literature showed negative results, EBV has been detected in large subsets of infiltrating breast cancer (Labrecque *et al.*, 1995; Fina *et al.*, 2001). However, Arbach *et al.* (2006) have detected EBV by quantitative PCR in whole tumors and microdissected tumors cells. In this study real time Q-PCR was applied to measure the EBV genome from frozen breast cancer biopsies; these findings show that EBV DNA was detected in more than the half of tumors (78.12%) and the viral load is highly variable from tumor to another and within each tumor. The present study is in accordance with the study of Arbach *et al.* (2006) and shows that EBV frequency in Algerian breast cancer was not different from French patients. According to these authors, detection of viral DNA in breast cancers has largely depended on the use of PCR techniques with 35 to 50 cycles of amplification or in combination with detection by other techniques (Hermann and Niedobitek, 2003). The evaluation of EBV in breast cancer has disclosed very low viral loads (Fina *et al.*, 2001) which have been defined as negative EBV

(Perkins *et al.*, 2006). In contrast, Labrecque *et al.* (1995) have detected the EBV genome in a small proportion of tumor cells by *in situ* hybridization assay in 12/19 PCR positive frozen tissue.

The copy number estimated by Luqmani and Shousha (1995) was 20-500 copies per microgram of DNA tissue by comparing with EBV containing plasmid. If one suppose that 1 cell contains 6 pg of DNA, this number would correspond to 0.003-0.08 copies per 1000 cells. Murray (2006) measured, by Q-PCR, two, twelve and five samples, respectively with less than 0.1, 0.1-0.9 and 1-7 copies of the EBV genome per 1000 cells. Not only similar results were found but also have reported some samples with 2 to 109 copies per 1,000 cells. In this study, EBV genomes were detected in various copy numbers in more than half of the breast cancer specimens analyzed, with high viral load compared to that found by Murray (2006).

CONCLUSION

To conclude, it is well known that breast cancer may comprise a heterogeneous population of breast cancer cells. Similarly, the distribution of EBV genomes in the same tumor was demonstrated to be very heterogeneous. Because EBV is detected in only some breast cancer cells, it is unlikely to be a primary etiologic agent. Hermann and Niedobitek (2003) inquired the implication of EBV in breast cancer, as the virus was detected in only a subset of tumor cells. However, EBV might have a role in earlier steps causing carcinogenesis. Alternatively, infection with EBV at a late state of tumor development might enhance oncogenic properties, such as invasiveness, angiogenesis and metastasis. For example, it has been shown that LMP-1 induces matrix metalloproteinase 9 (MMP9) and Vascular Endothelial Growth Factor (VEGF) (Wakisaka *et al.*, 2002), also induces and causes release of FGF-2 in human epithelial cells. Patients with breast cancer associated with EBV, have more aggressive form of the disease. Virus detection might be relevant to planning the treatment cycle. The possibility that EBV may be involved in the pathogenesis of breast cancer has to be taken into account. Therefore, viral infection appears as one of the main preventable cancer risk factors.

To summarize this study and because EBV genome is present in more than 78% of samples, it would be more sensible to widen the study on a number of frozen biopsy much more important, it is also necessary to look at the expression of the viral genes (EBNA and LMP) in our tumors. Whether EBV might have a role in breast cancer development or progression, we need now to be promptly investigated with appropriate cells.

ACKNOWLEDGMENTS

This study was supported by the Algerian Fellowship Program. I would like to thank the Ministry of Higher Education and Scientific Research which funded this work. I also thank, the Algerian Consulate in Paris who worked closely with the home laboratory. Dr. Irene Joab, actually retired, is gratefully acknowledged for receiving me in the Unit.

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