

Testing the Involvement of Viral Infections in Breast Cancer Diseases

Ali S. Gad Al Karim ^{1*}, Abd El Moneam M. Sharaf ¹, Mustafa Abd El-Fadeel ², Mohamed S. Al Shinawi ³, Hesham M. El-Mahdy ¹

¹ Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Nasr City, Cairo, Egypt

² Naval American Medical Research Unit-3 (NAMRU-3), Cairo, Egypt

³ General Surgery Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt

*aliashandawely@yahoo.com

Abstract: The most common cancer worldwide among women is breast cancer. The initiation, promotion, and progression of this cancer result from both internal and external factors. One of the reasons of the breast cancer causes is hormonal responsible viruses. In this study, we screened for anti-EBV, anti-HPV and anti-MMTV IgG antibodies in peripheral blood of 40 invasive ductal carcinoma (IDC) and 10 non-IBC patients. In addition, we screened for EBV, HPV-DNA and MMTV RNA in postsurgical cancer and non-cancer breast tissues of IBC and non-IBC patients, were Epstein-Barr Virus (EBV) 67.5%, Human Papilloma Virus (HPV) 50%, and Mouse Mammary Tumor Virus (MMTV) 57.5%. Our results reveal that IBC patients are characterized by a statistically significant increase in EBV, HPV and MMTV IgG antibody titers compared to non-IBC patients. EBV, HPV-DNA and MMTV RNA was significantly detected in cancer tissues than in the adjacent non-carcinoma tissues of IBC and IDC, and IBC cancer tissues were significantly more infected with EBV, HPV-DNA and MMTV RNA compared to IDC. Etiology and causality of EBV, HPV, MMTV infection with IBC now needs to be rigorously examined.

[Gad Al Karim AS, Sharaf AM, Abd El-Fadeel M, Al Shinawi MS, El-Mahdy HM. **Testing the Involvement of Viral Infections in Breast Cancer Diseases.** *Cancer Biology* 2014;4(3):11-21]. (ISSN: 2150-1041). <http://www.cancerbio.net>. 2

Keywords: BC; EBV; HPV; MMTV; ER; PR

1. Introduction

Breast cancer is a disease in which certain cells in the breast become abnormal and multiply without control or order to form a tumor. The primary risk factors for breast cancer are female sex and older age. Other potential risk factors include: lack of childbearing or breastfeeding, higher hormone levels, diet and obesity (Santoro et al., 2009). Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths. Recent statistics revealed that approximately 207,090 new cases of invasive breast cancer and 40,230 deaths from disease were diagnosed in USA in 2010 (Hernandez et al., 2010).

In Egypt, breast cancer is number one among women constituting about 38.6% of female cancer cases (National cancer registry program of Egypt, 2010) and it was found that it is more prominent among young premenopausal Egyptian women characterized by poor prognosis and low survival rate (Omar et al., 2003).

Most of Egyptian patients are diagnosed at advanced stage (II and III) (Gharbiah population-based cancer registry, 2007 and National cancer registry program of Egypt, 2010) and most cases possess positive axillary lymph node metastasis about 70.6% at the time of diagnosis while only 29.4% are free of metastasis (Nouhet et al., 2004). Besides, IBC

occurs in approximately 1-6% of all breast cancers in the U.S (Hance et al., 2005 and Bastawisy et al., 2012), in Egypt it occurs in approximately 10% of all breast cancers (Spencer et al., 2011), which reflects the invasive properties of breast cancer in Egyptian patients.

As a subtype, inflammatory breast cancer (IBC) is the most aggressive apparition of primary epithelial breast cancer and it belongs to the group of locally advanced breast cancer (LABC) characterized by a variety of clinical presentations including large tumors, extensive nodal involvement and direct involvement of the skin or chest wall. IBC is a clinic pathological entity characterized by the rapid onset of swelling and often enlargement of the breast. Although the overlying skin remains intact, it displays erythema often combined with 'peaud' orange', local tenderness, induration and warmth. All these changes can occur to a variable degree. Critical in this definition is the rapidity with which these occur (Kleer et al., 2000; Anderson et al., 2003; Dawood et al., 2011 and Nouh et al., 2011). According to the population-based cancer registry in Egypt, IBC cases represent around 11.1% of breast cancer cases between Egyptian patients and this percentage is on the rise (Soliman et al., 2009).

The aggressive nature of IBC, in addition to the unique molecular and epidemiological characteristics supports the hypothesis that IBC has its

own characters rather than being a subtype of locally advanced breast cancer (Dirix *et al.*, 2006; Vermeulen *et al.*, 2010 and Dawood *et al.*, 2011). For instance IBC can be distinguished from the LABC by the presence of numerous dermal tumor emboli in the papillary and reticular dermis of the skin overlying the breast (Bonnier *et al.*, 1995; Tomlinson *et al.*, 2001; Kleer *et al.*, 2001 and Dawood *et al.*, 2011).

The rapid onset, the misdiagnosis and lack of enough biological markers of IBC make it very critical to introduce new molecular and biological markers to accurately and early diagnose the disease. One of the reasons of the breast cancer causes is hormonal responsible viruses. The main candidate viruses are human papilloma virus (HPV), mouse mammary tumor virus (MMTV) and Epstein-Barr virus (EBV). Each of these viruses has known oncogenic potential and all have been identified in normal and malignant human breast tissues. The past priority has been to confirm the identity of these viruses in breast tumors. The new priority is to determine whether or not they are causal rather than innocuous passengers invading pre-existing malignant tissues. The formal search for the causes of human breast cancer began over 100 years ago. Despite cancer research expenditures exceeding \$100 billion US dollars during the past 30 years, that hunt, and the basic causes of breast cancer, remain elusive (Kolata, 2009). However, sound progress has been achieved.

The purpose of this study is Determine if that-oncogenic hormonal responsible viruses "Epstein Barr virus (EBV), high risk human papilloma virus (HPV), and mouse mammary tumor viruses (MMTV)" may have co-exist roles in human breast cancer.

2. Patients and Methods

2.1. Patients and Controls Samples

Patients were clinically and pathologically diagnosed as breast cancer patients prior to enrollment during the period of January 2010 to January 2012, from the breast clinics of Ain Shams University Hospitals, and the Faculty of Medicine of Ain Shams University, Cairo, Egypt. Serum and tissue samples were collected from forty patients enrolment will include suspect cases of having breast cancer of viral (n=40) and non-viral (n=10) origins. Ten normal healthy women patients (Inflammatory BC) collected from different places of Egypt. A series of 40 patients treated with surgical excision for primary breast carcinoma. As well as ten samples ones from normal controls. Tissues examined included both mastectomy and excision biopsy specimens. Inclusion in the series required that there be sufficient fresh tumour material available for all the assays to be performed. Tumor tissue was subdivided with provision for both

hormone receptor studies and routine diagnostic preparations. Other samples were snapfrozen and stored at - 70°C. For each patients tumor block, two (10µ thick sections) were cut into a sterile eppendorf tube for subsequent DNA and/or RNA extraction. As well as Ten ml of peripheral blood was collected from each patient and healthy volunteers for Serology detections.

2.2. Serology Tests

EBV ELISA for IgG antibodies against EBNA-1 was performed on serum samples of cases and controls using a commercial ELISA kit (Serion Immunodiagnostics, Wurzberg, Germany). The test was carried out using manufacturer's instructions. The assay run in this study met the quality control. The kit cannot reliably measure IgG antibody levels above 110 IU/mL; hence all high values are truncated at this point. Due to limited resources we performed serology in cases and half of randomly selected controls.

HPV ELISA for IgG antibodies against HPV-16 L1 IgG was performed on serum samples of cases and controls using a commercial ELISA kit (Alpha diagnostic international, Texas, USA). The test was carried out using manufacturer's instructions.

MMTV ELISA for quantitative measurement of WNT1 in was performed on serum samples of cases and controls using a commercial ELISA kit (Antibodies online.com). The test was carried out using manufacturer's instructions.

2.3. PCR Tests

DNA was extracted from between 50-200 mg of homogenized tissue using standard procedures (phenol/chloroform and ethanol precipitation) after digestion with proteinase K and lysis buffer (0.2% sodium dodecyl sulfate in 10mM Tris, 10mM EDTA, 50mM NaCl) at 37°C overnight.

2.3.1. PCR for HPV

HPV consensus primers, MY09 (5'CGTCCMARRGGAWACTGATC-3') and MY11 (5'-GCMCAGGGWCATAAYAATGG-3') where M = A + C, R = A + G, W = A + T and Y = C + T, were used in the PCR assay to amplify an approximately 450-bp fragment from the L1 regions of HPV-16 and 18 (D'Costa *et al.*, 1998). The final 50µl PCR mixture contained 10-µl sample, 25µl PCR Master Mix (Promega), 3mM MgCl₂, 20pmol of each primer. Amplifications were performed with the following cycling profile: incubation at 94°C for 5 min followed by 40 cycles of 1-min denaturation at 95°C, 1-min annealing at 55°C, and 1-min elongation at 72°C. The last cycle was followed by a final extension of 10 min at 72°C.

2.3.2. PCR for MMTV

PCR was carried out using primers 2N(5'-CCTACATCTGCCTGTGTTAC-3') and 3N (5'-ATCTGTGGCATACTAAAGG-3') which amplify an approximately 255-bp segment from the MMTV *env* gene (Wang *et al.*, 1995). The amplification reaction was carried out with 100pmol of primers using PCR BEADS from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). Each reaction contained in 25 ml, the following components: (a) 1.5 units of *Taq* DNA polymerase; (b) 10mM Tris-HCl (pH 9.0 at room temperature); (c) 50mM KCl; (d) 1.5mM MgCl₂; and (e) 200mM of each of the four nucleotides. Thermocycling was performed in a DNA cycler (Perkin-Elmer) by denaturation at 94°C for 1–5 min annealing at 55°C for 1 min, and elongation at 72°C for 1–5 min for 35 cycles.

2.3.3. PCR for EBV

PCR for EBV DNA was performed according to the method of Hashimoto *et al.* (1995) with some modifications. The PCR mixture contained 1µg DNA, 5µl of 10× *Taq* polymerase buffer (100mM Tris-HCl, pH 8.8, 500mM KCl, 15mM MgCl₂, and 1% Triton X-100), 200µmol/l of each deoxynucleotide, 25pmol of EBV primers (5'-CACTTTAGAGCTCTGGAGGA-3' and 5'-TAAAGATAGCAGCAGCGCAG-3'), and 1.25 units of *Taq* polymerase in a final volume of 50µl. After initial incubation for three minutes at 94°C, 40 PCR cycles were performed which involved denaturing at 94°C for one minute annealing at 57°C for 0.5 minute, and extension at 72°C for 1.5 minute. The amplified product was 153 base pair fragment of the Bam HI "W" region of the EBV *Bam*HI W internal repetitive fragment.

2.4. Electrophoresis analysis of PCR product

PCR-amplified DNA fragments were separated by agarose gel electrophoresis in 1.5 % agarose (Seakem LE, FMC, Bio products, Cat. # 50004) minigel in 0.5X TBE buffer (Tris-borate-EDTA, 90mM Tris acetate, 90mM boric acid, 2mM EDTA, pH 8.0) using 100bp DNA ladder (Promega, Cat # G2101). The gels were visualized with UV light after staining for 10 min with 10 µg/ml of ethidium bromide [2,7-Diamino-10-ethyl-9 phenyl phenanthridinium bromide; homidium bromide. (Sigma, Cat. # E7637)] (Sambrook *et al.*, 1989), and visualized with UV illumination using Gel Documentation System (Gel Doc 2000 BIORAD 1000/115 V ~ 50/60 Hz-150 VA). The expected size of the PCR products was ~300bp, ~371bp and ~371bp for HPV, MMTV and EBV, respectively.

3. Results

A total of 40 female breast cancer patients as well as 10 control healthy patients with fibrocystic inflammation and their surgically obtained tissue specimen were studied. Among the viral gene-positive breast cancer samples, 7 and 5 cases (17.5%), (12.5%) were positive for only one virus HPV and MMTV respectively, 9, 14 cases (22.5%), (35%) were positive for two viruses EBV with HPV and EBV with MMTV respectively, 4 cases (10%) were positive for three viruses (EBV, HPV and MMTV) (Table 1). The clinic-pathological characteristics of the breast cancer patients are summarized in table (2). All Cancer tissues (n=40) were screened for the presence of three different viruses using ELISA and PCR analysis. Among the 40 breast cancer samples, twenty seven (67.5%) were positive for EBV, twenty (50%) were positive for HPV, and twenty three (57.5%) were positive for MMTV while in non-breast cancer patients "Healthy" it was detected in only one case with EBV (10%) in PCR detection. While it was twenty nine were positive of EBV (72.5%), 21 (52.5%) were positive for HPV, and 23 (57.5%) were positive for MMTV and it was also detected in only one case with EBV (10%) by ELISA non-breast cancer patients "Healthy".

Table 1. Frequency of single or multiple viruses (EBV, HPV and MMTV) in the same breast cancer specimen (invasive ductal carcinomas and invasive lobular carcinomas) and epithelial cells from 10 fibrocystic disease specimens using PCR

	Breast Cancer N = 40	Normal breast N = 10
EBV	0(0%)	1(10%)
HPV	7(17.5%)	0(0%)
MMTV	5(12.5%)	0(0%)
EBV+ HPV	9(22.5%)	0(0%)
EBV+ MMTV	14(35%)	0(0%)
HPV+MMTV	0(0%)	0(0%)
EBV+HPV+MMTV	4(10%)	0(0%)
NO virus	1(2.5%)	1(10%)

Among the 10 viral DNA-and or RNA positive specimens of the non-breast cancer controls, only one type of virus was found in the normal and tumor specimen groups.

3.1. Relation between involvement viral infections in breast cancer and Clinical findings and histopathological study

3.1.1. Age of patients

The average age of patients with fresh frozen breast cancer was EBV positive (48.5 years) and

MMTV positive (50.7 years) and was significantly younger than patients with EBV negative (43.6 years) and MMTV negative (41.9 years) breast cancers, However HPV negative (50.8 years) was significant in older age than patient with HPV positive (43.2 years). According to the patients age and menopausal status for invasive ductal and or lobular carcinoma (25/40) cases 62.5% were positive for virus individual or all. It's showed the age from $\geq 20 - \leq 30$ (2/40) cases 5%, (1/40) cases 2.5%, (2/40) cases 10% and (0/40) cases 0.0% positive for EBV, HPV and MMTV respectively. While $\geq 31 - \leq 50$ was (24/40) cases 60%, (13/40) cases 32.5% , 13/40 cases (32.5%) and (12/40) cases 30% positive for EBV, HPV and MMTV respectively, and the age from $\geq 51 - \leq 80$ showed (14/40) cases 35% was (12/40) cases 30%, (5/40) cases 12.5% and (11/40) cases 27.5% positive for EBV, HPV and MMTV, respectively.

3.1.2. Breast cancer grade

There is a significant increase in grade of EBV, HPV and MMTV in grade II tumor than other of positive invasive breast cancer specimens, It was in only two out of forty cases one it was HPV post (1/40) 2.5% other one it was negative to all three selected viruses, while in grade II was (9/40) cases 47.5%, (10/40) cases 25% and (12/40) cases 35% shows positive in grade II for EBV, HPV, and MMTV respectively, in grade III it was (9/40) cases 22.5%,

(9/40) cases 22.5% and (11/40) cases 27.5% shows positive for EBV, HPV, and MMTV, respectively.

3.1.3. Steroid hormone receptor

There are differences in estrogen and progesterone receptor expression between viral positive and negative fresh frozen breast cancer specimens, As regards hormonal receptor study, ER showed positive immunoreactions for (16/27) cases 59.2%, (5/20) cases 25% and (19/23) cases 82.6% positive for EBV, HPV and MMTV respectively. As regards positive immunoreactions for PR. it was (16/27) cases 59.2%, (5/20) cases 25% and (20/23) cases 86.9% positive for EBV, HPV and MMTV, respectively.

3.1.4. Lymph Node

As regards Lymph Node (LN) status, out of the forty cases showed positive nodal metastases (31/40) cases 77.5%, it was (17/40) cases 42.5% had 1-3 LN involvement (12/17) cases 70.5%, (11/17) cases 64.7% and (6/17) cases 35.3% cases was positive for EBV, HPV and MMTV respectively, While (14/40) cases 35% had ≤ 4 , (9/14) cases 64.3%, (5/14) cases 42.8% and (13/14) cases 92.8% was positive for EBV, HPV and MMTV respectively, while only (9/40) cases 22.5% showed negative nodal metastases., (6/9) cases 66.6%, (4/9) cases 44.4% and (4/9) cases 44.4% for EBV, HPV and MMTV, respectively.

Table 2. Patients and prognostic factor

Characteristics		IBC Patients (n=40)
Age	Range	29-67
	Mean± SD	45.7
Tumor Size (Cm ³)	≤ 10	3
	10-19	11
	20-9	9
	≥ 30	17
Axillary Lymph Node Metastasis	Neg.	9
	1-3	17
	≥ 4	14
Menopausal Status	Postmenopausal	18
	Premenopausal	22
Steroid Hormone	ER- Positive	21
	ER- Negative	19
	PR- Positive	22
	PR-Negative	18
Tumor histology	Ductal	27
	Lobular	13
Tumor Grade (SBR)	G-I	2
	G- II	20
	G-III	18

3.1.5. Tumor Histology

As regards patients with BC, Out of forty cases it was showed (27/40) cases 67.5% had invasive ductal carcinoma, Out of twenty seven cases it was (19/27) cases 70.3%, (12/27) cases 44.4% and (18/27) cases 66.7% cases positive for EBV, HPV, and MMTV respectively, while invasive lobular was diagnosed in thirteen out of forty cases (44.3%). Out of thirteen cases it was (8/13) cases 61.5%, (8/13) cases 61.5% and (5/13) cases 38.5% positive for EBV, HPV, and MMTV, respectively.

3.1.6. EBV-DNA detection and prognostic factors

There are association was observed between steroid receptor expression and viral positive or negative, association was observed between EBV-DNA detection and patients' age and menopausal status it was significant in younger age, However significant associations were detected between the presence of EBV-DNA and other poor prognostic factors. Most of the EBV-DNA positive BC was significantly associated with positive nodal status, where 21/27 cases 77.7% showed nodule tumor status -positive LN involvement. In spite of the small number of invasive lobular carcinoma included in this study there was a significant correlation between this histological type of poor prognosis and EBV-DNA detection rate where (8/27) cases 29.6% of them were positive for EBV-DNA compared to (19/27) cases 70.3% detection rate in invasive lobular and ductal carcinoma A significant correlation was found between EBV DNA detection rate in BC and high tumor grade of invasive ductal carcinoma; 18/27 cases (66.6%) association with grade III versus (9/27) cases 33.4% with grade II (Table 3). EBV-DNA was not detected in all BC cases with tumor size < 2 cm. On the other hand most positive cases for EBV-DNA were of tumor size >10 cm³, however the difference was statistically significant, <10 cm³ (1/27) cases 3.7%, 10-19 cm³ (8/27) cases 29.6%, 20-29 cm³ (4/27) cases 14.8% and >30 cm³ (14/27) cases (51.8%), respectively.

3.1.6.1. Detection of EBV in the control group

A significant statistical difference was detected between patients with BC and the control group as regard expression of EBV; was detected in only one specimen of the control group indicating that EBV is significantly restricted to BC.

3.1.6.2. The correlation between EBNA-1 and EBV-DNA detection in BC

The results of EBNA-1 serological assay detection and PCR analysis significantly correlated with each other. All PCR EBV-positive BC were EBNA-1 positive. Only 2 EBNA1 positive tumors were not detected by PCR-EBV.

3.1.7. HPV-DNA detection and prognostic factors

There are no association was observed between steroid receptor expression, patient's age and viral positive or negative. While association was observed between HPV-DNA detection and menopausal status, it was insignificant in age between $\geq 20 - \geq 30$ years where (2/20) cases (10%), $\geq 31 - \leq 50$ years, Where (13/20) cases (65%) and $\geq 51 - \leq 80$ was (13/20) cases (56.5%), respectively. However significant associations were detected between the presence of HPV-DNA and other poor prognostic factors. Most of the HPV -DNA positive BC were significantly associated with positive nodal status, where (16/20) cases (80%) showed nodule tumor status -positive LN involvement. In spite of the number of invasive lobular carcinoma included in this study there was a significant correlation between this histological type of poor prognosis and HPV-DNA detection rate where (8/20) cases 40% of them were positive for HPV-DNA compared to (12/20) cases 35% detection rate in invasive ductal carcinoma. A significant correlation was found between HPV-DNA detection rate in BC and high tumor grade of invasive ductal carcinoma; (10/20) cases 50% association with grade III versus (9/20) cases 45% with grade II and (1/20) cases 5% with grade I (Table 4). HPV -DNA was not detected in all BC cases with tumor size < 2 cm. On the other hand most positive cases for HPV-DNA were of tumor size >2 cm³, however the difference was statistically significant; <10 cm³ (1/20) cases (5%), 10-19cm³ (5/20) cases 25%, 20-29 cm³ (9/20) cases 45% and ≥ 30 cm³ (5/20) cases (25%).

Table 3. Correlation between EBV by ELISA and PCR and prognostic factors in the breast cancer patients

Prognostic Factors	Criteria	Detection of (EBV) by ELISA	Detection of (EBV) by PCR
Cases		29	27
Age	$\geq 20 - \leq 30$	1	1
	$\geq 31 - \leq 50$	16	14
	$\geq 51 - \leq 80$	12	12
Tumor Size, (cm³)	≤ 10	1	1
	10-19	8	8
	20-29	5	4
	≥ 30	15	14
Axillary Lymph Node Status	Negative	7	6
	1-3	12	12
	≥ 4	11	9
Menopausal Status	Post.	16	16
	Pre.	13	11
Steroid hormone receptors	ER Pos.	16	16
	ER Neg.	12	11
	PR Pos.	17	16
	PR Neg.	11	11
Tumor Histology	Ductal	20	19
	Lobular	9	8
Tumor Grade (SBR)	I	1	0
	II	10	9
	III	18	18

Table 4. Correlation between HPV by ELISA and PCR and prognostic factors in the breast cancer patients

Prognostic Factors	Criteria	Detection of (HPV) by ELISA	Detection of (HPV) By PCR
Cases		21	20
Age	$\geq 20 - \leq 30$	2	2
	$\geq 31 - \leq 50$	14	13
	$\geq 51 - \leq 80$	5	5
Tumor Size, (cm³)	≤ 10	2	1
	10-19	5	5
	20-29	9	9
	≥ 30	5	5
Axillary Lymph Node Status	Negative	5	4
	1-3	11	11
	≥ 4	5	5
Menopausal Status	Post.	6	6
	Pre.	15	14
Steroid hormone receptors	ER Pos.	5	5
	ER Neg.	16	15
	PR Pos.	5	5
	PR Neg.	16	15
Tumor Histology	Ductal	12	12
	Lobular	9	8
Tumor Grade (SBR)	I	2	1
	II	9	9
	III	10	10

3.1.7.1. Detection of HPV in the control group

A significant statistical difference was detected between patients with BC and the control group as regard expression of HPV; was not detected in any specimen of the control group indicating that HPV is significantly restricted to BC.

3.1.7.2. The correlation between HPV-16 L-1 IgG and HPV-DNA detection in BC

The results of HPV-16-L-1 serological assay detection and PCR analysis significantly correlated with each other. All HPV PCR-positive BC were HPV-16-L-1 positive.

3.1.8. MMTV-RNA detection and prognostic factors

While association was observed between MMTV-RNA detection and menopausal status it was significant. However significant associations were detected between the presence of MMTV-RNA and other poor prognostic factors. Most of the MMTV-RNA positive BC were significantly associated with positive nodal status, where (19/23) cases (82.6%) showed nodule tumor status -positive LN involvement. In spite of the invasive lobular carcinoma included in this study there was a significant correlation between this histological type of poor prognosis and MMTV-RNA detection rate where (5/23) cases 21.7% of them were positive for invasive lobular carcinoma compared to (18/23) cases 78.3% detection rate in invasive ductal carcinoma respectively. A significant correlation was found between MMTV-RNA detection rate in BC and high tumor grade of invasive ductal carcinoma; (12/23) cases 52% association with grade III versus (11/23) cases 48% with grade II respectively Table (5). MMTV-RNA was not detected in all BC cases with tumor size $< 2 \text{ cm}^3$. On the other hand most positive cases for MMTV-RNA were of tumor size $\geq 2 \text{ cm}^3$, however the difference was statistically significant; $\leq 10 \text{ cm}^3$ (2/23) cases 8.7%, $10-19 \text{ cm}^3$ (4/23) cases 17.4%, $20-29 \text{ cm}^3$ (1/23) cases (3.4%) and $\geq 30 \text{ cm}^3$ (16/23) cases 69.5%, respectively (Table 5).

Table 5. Correlation between MMTV by ELISA and PCR and prognostic factors in the breast cancer patients

Prognostic Factors	Criteria	Detection of (MMTV) by ELISA	Detection of (MMTV) by PCR
Cases		23	23
Age	$\geq 20 - \leq 30$	0	0
	$\geq 31 - \leq 50$	12	12
	$\geq 51 - \leq 80$	11	11
Tumor Size, (cm³)	≤ 10	2	2
	10-19	4	4
	20-29	1	1
	≥ 30	16	16
Axillary Lymph Node Status	Negative	4	4
	1-3	6	6
	≥ 4	13	13
Menopausal Status	Post.	15	15
	Pre.	8	8
Steroid hormone receptors	ER Pos.	19	19
	ER Neg.	4	4
	PR Pos.	20	20
	PR Neg.	3	3
Tumor Histology	Ductal	18	18
	Lobular	5	5
Tumor Grade (SBR)	I	0	0
	II	11	11
	III	12	12

3.1.8.1. Detection of MMTV in the control group

A significant statistical difference was detected between patients with BC and the control group as regard expression of MMTV; was not

detected in any specimen of the control group indicating that MMTV is significantly restricted to BC.

3.1.8.2. The correlation between MMTV-WNT-1 Ag and MMTV-RNA detection in BC

The results of MMTV-WNT-1 Ag serological assay detection and PCR analysis significantly correlated with each other. All PCR MMTV-positive BC were MMTV-WNT-1 Ag positive.

4. Discussion

We have demonstrated that EBV, HPV, and MMTV viral sequences may all be present as individual or multiple viruses in human breast cancer and rare in normal fibrocystic disease of breast cancer. These three viruses appear to be present in a significantly higher proportion of breast cancers as compared to fibrocystic disease of breast cancer women.

The three most studied viruses that could possibly cause breast cancer in humans are: Mouse Mammary Tumor Virus (MMTV), Epstein-Barr virus (EBV) and Human Papilloma Virus (HPV) (Lawson, 2001). MMTV and EBV occur in 37% and 50% of breast cancer cases, respectively (Mant et al., 2004).

Over the past decade, the EBV, HPV and MMTV association with BC has been constantly debated despite the well-documented presence of EBV genetic material in up to 66%, 48% and (62%) respectively of breast tumors (Horiuchi et al., 1994; Kan et al., 2005 and Wang et al., 2003). This might be due in part to epidemiological variation in EBV infections, like difference in the age at which the studied patients had acquired primary EBV infection; as populations with higher incidence rates of BC correspond to those with higher likelihood of delayed primary EBV infection (Yasui et al; 2001). In addition, this controversy might be due to differences in the methodologies used for detecting the virus and different EBV-derived proteins or nucleic acid analyzed. The identification of EBV in this current study, using serological techniques by ELISA and standard PCR, is consistent with the findings of others.

In the present study by using PCR targeting sequences specific for the Bam HI-W region of EBV. Among several studies using PCR for detecting EBV in BC, prevalence is noticed to be highest when PCR targeted Bam HI-W or EBERs (Epstein-Barr Encoded RNA) sequences, more moderate when PCR targeted LMP1 or EBNA-4 gene, and lowest in examination of EBNA-1 gene; indicating the importance of the PCR target on the extent of the association (Glaser et al.; 2004).

EBV-DNA has been detected in 67.5% of BC samples. Indeed, EBV has been identified most consistently using PCR as in studies done by (Horiuchi et al., 1999), (Labrecque et al; 2001),

(Luqmani et al ; 1995), (Bonnet et al.; 1999), (Fina et al.; 2001), (Preciado et al.; 2005), (Tsai et al.; 2005), and (Perkins et al.; 2005) with respective frequencies 66%, 20%, 40%, 51%, 31.8%, 35%, 45.2% and 46%.

Breast tissue from normal women as controls has been used in only four out of the 22 studies, (Perrigou et al., 2005), normal breast tissue from sites adjacent to the tumor were used as normal controls; such tissues are more likely to carry suspect viruses than normal tissue sourced from normal women). EBV genetic material was rarely identified in normal control breast tissues in contrast to its identification in up to 51% of tumor tissues. While this major difference between cases and controls is strongly suggestive of a role for EBV in breast cancer, the identification by Junker and colleagues of EBV genetic material in nearly half of the milk samples from normal women (Junker et al., 1991), suggests a need for caution when drawing any conclusions.

Bonnet and colleagues was detect (10%) (3/30) of EBV of patients control, it compatible for our study (10%) (1/10). The fact that EBV can be transferred by direct contact from lymphocytes to breast epithelial cells suggests that a role in breast carcinogenesis is possible (Speck et al., 2000).

From these results we can suggest that EBV may play a role in breast cancer oncogenesis but it is unlikely to be a primary etiological agent as EBV is only detected in some breast cancer cells. Instead, EBV mostly acts in concert with other co-factors. It may alter the behavior of already transformed cells so that they acquire a more aggressive phenotype. This hypothesis is supported by the observation that EBV-associated breast cancers are more commonly aggressive than other breast cancers (Bonnet et al.; 1999 and Murry et al.; 2003).

The recent identification of HPVs by de Villiers and colleagues, Damin and colleagues and Kan and colleagues in breast tumors (de Villiers et al., 2005, Damin et al., 2004 , Kan et al., 2005), has established HPVs as strong candidate oncoviruses for breast cancer. de Villiers and colleagues have shown that breast nipple tumors in which HPVs have been identified have histological characteristics typical of HPV-associated human cancers (such as the cervix) (de Villiers et al., 2005). de Villiers and colleagues identified a range of HPV types in 25 out of 29 samples of breast carcinoma (de Villiers et al., 2005). Damin and colleagues identified HPV types 16 and 18 in 24.75% of breast tumors (n = 105) but not in normal controls (Damin et al., 2004). Kan and colleagues identified HPV-18 in 48% of DNA extracted from breast tumors (Kan et al., 2005), where our study identified HPV-16 in 50% of the cases.

The presence of HPV DNA genes in breast tumors has been identified in ten out of 12 studies,

including every study conducted since 1999. Normal breast tissue controls were available for four of these studies. In these four studies, there were 215 cases, and HPV gene sequences were identified in 51 (23.7%). There were 89 controls, and HPV sequences were identified in one (1.1%).

The HPV are accepted as carcinogenic in human cervical and anogenital cancer. The suspicion that HPV may also play a role in human breast cancer is based on the identification of HPV of high oncogenic risk (16, 18, 31, 33, and 35) in these tumors, and in the immortalization of the human breast normal cells. The controversy surrounding the HPV involvement with breast cancer can occur due to the difficulty to find the virus in the specimens, contrasting with the facility for detecting cervical cancer (Lindel et al., 2007).

The identification of HPV in this current study, using serological techniques by ELISA and standard PCR, is consistent with the findings of others. In the present study using PCR targeting sequences specific for the *HPV L1 gene* -DNA has been detected in 50% of BC samples. Indeed, HPV has been identified most consistently using PCR as in studies done by (Yu et al., 2000), (Li et al 2002), (Widschwendter et al., 2004), (Kan et al., 2005), (Akil et al., 2008), (HeQ et al., 2009), (Aceto et al., 2010), (Antonsson et al., 2011) it was respectively frequencies by PCR detection (43%)(14/32), (68%)(19/28), (64%)(7/11), (48%)(24), 61%)(69/113), (60%)(20/24), (60%)(3/5), (50%)(27/5) amongst these specimens have been co-infected with more than one HPV type.

Wang et al. (1998) identified mouse mammary tumor virus (MMTV) mRNA in breast cancer specimens, but not in normal breast tissues. Indik et al. (2007) demonstrated that MMTV rapidly spreads in cultured human breast cells, ultimately leading to the infection of all the cells in culture, thus providing further evidence that human cells are compatible hosts for MMTV. Their observations further suggest that cross-species transmission of MMTV is generally possible and strengthens the contention that MMTV might be an etiological agent involved in human breast carcinogenesis. That is very similar to our result for MMTV in control healthy patients,

5. Conclusion

(i) EBV, HPV and MMTV gene sequences are present and co-exist in many human breast cancers, (ii) The presence of these viruses in breast cancer is associated with young age of diagnosis and possibly an increased grade of breast cancer and (iii) EBV and HPV may collaborate in some breast cancers.

6. Ethics Statement

For patient recruitment, Institutional Review Board (IRB) approval was obtained from the ethics committee of Ain-Shams University. All patients signed a consent form before participating in the study. Patients were clinically and pathologically diagnosed as breast cancer patients prior to enrollment during the period of January 2010 to January 2012, from the breast clinics of Ain Shams University Hospitals, and the Faculty of Medicine of Ain Shams University, Cairo, Egypt.

Acknowledgment

Grant thanks to Prof. Muna M. Mustafa and her team work, Head of Cancer Biology Research Lab (CBRL), Faculty of Science, Cairo University, for continuous help and support. Authors acknowledge the Dr. Ahmed R. Sofy Assistant Prof. of Virology and Bioinformatics (Microbiology Department, Faculty of Science, Al Azhar University).

Corresponding Author

Ali S. Gad Al Karim
Botany and Microbiology Department
Faculty of Science
Al-Azhar University, Nasr City, Cairo, Egypt
E-mail: aliashandawely@yahoo.com

References

1. Aceto GM, Solano AR, Neuman MI, Veschi S, Morgano A, Malatesta S et al (2010). High-risk human papilloma virus infection, tumor pathophenotypes, and BRCA1/2 and TP53 status in juvenile breast cancer. *Breast Cancer Res Treat.*; 122(3): 671-683.
2. Anderson WF, Chu KC, Chang S (2003). Inflammatory breast carcinoma and noninflammatory locally advanced breast carcinoma: distinct clinicopathologic entities?. *J. Clin. Oncol.*; 21: 2254-2259.
3. Bastawisy A, Gaafar R, Eisa S, Amira G, Helal M (2012). Inflammatory breast cancer: is it really a separate entity?. *Ecancermedalscience*; 6: 250.
4. Broadhurst MK, Lee RSF, Hawkins S, Wheeler TT (2005). The p100 EBNA-2 coactivator: a highly conserved protein found in a range of exocrine and endocrine cells and tissues in cattle. *Biochem. Biophys. Acta*; 1681: 126-133.
5. Chu PG, Chang KL, Chen YY, Chen WG, Weiss LM (2001). No significant association of Epstein-Barr virus infection with invasive breast carcinoma. *Am. J. Pathol.*; 159: 571-578 (2001).

6. Dawood S, Merajver SD, Viens P, Vermeulen PB, Swain SM, et al. (2011). International expert panel on inflammatory breast cancer: consensus statement for standardized diagnosis and treatment. *Ann. Oncol.*; 22: 515-523.
7. de León DC, Montiel DP, Nemcova J, Mykyskova I, Turcios E, Villavicencio V et al. (2009). Human papillomavirus (HPV) in breast tumors: prevalence in a group of Mexican patients. *BMC Cancer*; 9: 26-32.
8. Dirix LY, Van Dam P, Prové A, Vermeulen PB (2006). Inflammatory breast cancer: current understanding. *Curr. Opin. Oncol.*; 18: 563-571.
9. Fina F, Romain S, Ouafik LH (2001). Frequency and genome load of Epstein–Barr virus in 509 breast cancers from different geographical areas. *Br. J. Cancer*; 84: 783–790.
10. Gumus M, Yumuk PF, Salepci T, Aliustaoglu M, Dane F, Ekenel M, Basaran G, Kaya H, Barisik N, Turhal NS (2006). HPV DNA frequency and subset analysis in human breast cancer patients' normal and tumoral tissue samples. *J. Exp. Clin. Cancer Res.*; 25: 515–521.
11. Hance KW, Anderson WF, Devesa SS, Young HA, Levine PH (2005). Trends in inflammatory breast carcinoma incidence and survival: the surveillance, epidemiology, and end results program at the National Cancer Institute. *J. Natl. Cancer Inst.* 97: 966-975.
12. He Q, Zhang SQ, Chu YL, Jia XL, Wang XL (2009). The correlations between HPV16 infection and expressions of c-erbB-2 and bcl-2 in breast carcinoma. *Mol. Biol. Rep.*; 36(4): 807-812.
13. Hernandez BY, Green MD, Cassel KD, Pobutsky AM, Vu V, et al. (2010). Preview of Hawaii Cancer Facts and Figures 2010. *Hawaii Med; J.*; 69: 223-224.
14. Horiuchi K, Mishima K, Ohsawa M, Aozasa K (1994). Carcinoma of stomach and breast with lymphoid stroma: localization of Epstein–Barr virus. *J. Clin. Pathol.*; 47: 538-540.
15. Indik S, Günzburg WH, Kulich P et al (2007). Rapid spread of mouse mammary tumor virus in cultured human breast cells. *Retrovirology*; 4: 73.
16. Junker AK, Thomas EE, Radcliffe A, Forsyth RB, Davidson AGF, Rymao L (1991). Epstein–Barr virus shedding in breast milk. *Am. J. Med. Sci.*; 302: 220–223.
17. Kleer CG, van Golen KL, Merajver SD (2000). Molecular biology of breast cancer metastasis. Inflammatory breast cancer: clinical syndrome and molecular determinants. *Breast Cancer Res.*; 2: 423-429.
18. Kolata G (2009). Grant system leads cancer researchers to play it safe. *New York Times*, June 27, 2009. Available online at: <http://www.nytimes.com>
19. Köstler WJ, Schwab B, Singer CF, et al. Monitoring of serum HER-2/neu predicts response and progression-free survival to trastuzumab-based treatment in patients with metastatic breast cancer. *Clin. Cancer Res.*; 10: 1618-1624.
20. Labrecque LG, Barnes DM, Fentiman IS, Griffin BE (2001). Epstein–Barr virus in epithelial cell tumors: a breast cancer study. *Cancer Res.*; 55:39-45.
21. Lawson JS, Ginsburg WH, Whitaker NH. Viruses and human breast cancer. *Future Microbiol.*; 1(1): 33–51.
22. Lawson JS, Günzburg WH, Whitaker NJ (2006a) Viruses and cancer? Scrutinizing the molecular evidence. *Breast Cancer Res.*
23. Lawson, J.S.; Heads, J.; Glenn, W.K.; Whitaker, N.J. Breastfeeding, breast milk and viruses. *BMC Women's Health* 2007, 7, 17–20.
24. Li T, Lu ZM, Guo M, Wu QJ, Chen KN, Xing HP et al. p53 codon 72 polymorphism (C/G) and the risk of human papillomavirus-associated carcinomas in China. *Cancer*. 2002;95:2571-2576.
25. Luqmani Y, Shousha S. Presence of Epstein–Barr virus in breast carcinoma. *Int J Oncol* 1995;6:899–903.
26. Mant C, Hodgson S, Hobday R, D'Arrigo C, Cason J. A viral aetiology for breast cancer: time to re-examine the postulate. *Intervirology*. 2004; 47(1):2-13.
27. Murray PG, Lissaur D, Junying J et al.: Reactivity with a monoclonal antibody to Epstein–Barr virus nuclear antigen 1 defies a subset of aggressive breast cancers in the absence of the EBV genome. *Cancer Res.* 63,2338–2343 (2003).
28. Noh MA, Ismail H, El-Din NH, El-Bolkainy MN (2004) Lymph node metastasis in breast carcinoma: clinicopathological correlations in 3747 patients. *J Egypt NatlCancInst* 16: 50-56.
29. Omar S, Khaled H, Gaafar R, Zekry AR, Eissa S, et al. (2003) Breast cancer in Egypt: a review of disease presentation and detection strategies. *East Mediterr Health J* 9: 448-463.
30. Perkins RS, Sahm K, Marando C, et al. Analysis of Epstein–Barr virus reservoirs in paired blood and breast cancer primary biopsy specimens by real time PCR. *Breast Cancer Res* 2006;8(6) R70.
31. Perrigoue JG, den Boon JA, Friedl A, Newton MA, Ahlquist P, Sugden B: Lack of association between EBV and breast carcinoma. *Cancer Epidemiol. Biomarkers Prev.* 809–814 (2005).

31. Sambrook J, Fritsch EF, Maniatis T (1989). *Molecular cloning: A laboratory manual* 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
32. Santoro, E., DeSoto, M., and Hong Lee, J (February 2009). "Hormone Therapy and Menopause". National Research Center for Women & Families. (<http://www.center4research.org/2010/03/hormone-therapy-and-menopause/>).
33. Silva RG, Silva BB. No evidence for an association of human papillomavirus and breast carcinoma. *Breast Cancer Res Treat.* 2011; 125:261-264.
34. Simões PW, Medeiros LR, Pires PDS, Edelweiss MI, Rosa DD, Silva FR et al. Prevalence of Human Papillomavirus in Breast Cancer: A Systematic Review. *Int J Gynecol Cancer.* 2012;22(3):343-347.
35. Soliman AS, Banerjee M, Lo AC, Ismail K, Hablas A, et al. (2009) High proportion of inflammatory breast cancer in the Population-based Cancer Registry of Gharbiah, Egypt. *Breast J* 15: 432-434.
36. Speck P, Longnecker R: Infection of breast epithelial cells with Epstein–Barr virus via cell-to-cell contact. *J. Natl Cancer Inst.* 92, 1849–1851 (2000).
37. Spencer B, Banerjee M, Omar S, Khaled H, Anwar N, et al. (2011) Survival of inflammatory breast cancer patients compared to non-inflammatory breast cancer patients in Egypt. *Breast J* 17: 545-547.
38. Tsai JH, Tsai CH, Cheng MH, Lin SJ, Xu FL, Yang CC. Association of viral factors with non-familial breast cancer in Taiwan by comparison with non-cancerous, fibroadenoma, and thyroid tumor tissues. *J Med Virol* 2005;75(2):276–81.
39. Vermeulen PB, van Golen KL, Dirix LY (2010) Angiogenesis, lymphangiogenesis, growth pattern, and tumor emboli in inflammatory breast cancer: a review of the current knowledge. *Cancer* 116: 2748-2754.
40. Wang Y, Go V, Holland JF et al (1998) Expression of mouse mammary tumor virus-like env gene sequences in human breast cancer. *Clin Cancer Res* 4:2565–2568
41. Wang Y, Melana SM, Baker B et al.: High prevalence of MMTV-like env gene sequences in gestational breast cancer. *Med. Oncol.* 20, 233–236 (2003).
42. Widschwendter, A.; Brunhuber, T.; Wiedemair, A.; Mueller-Holzner, E.; Marth, C. Detection of human papillomavirus DNA in breast cancer of patients with cervical cancer history. *J. Clin. Virol.* 2004, 31, 292–297.
43. Yasui, Y.; Potter, J.D.; Stanford, J.L.; Rossing, M.A.; Winget, M.D.; Bronner, M.; Daling, J. Breast cancer risk and "delayed" primary Epstein-Barr virus infection. *Cancer Epidemiol. Biomarkers Prev.* 2001, 10, 9–16.

8/12/2014