Molecular characterization of viruses involvement in breast cancer patients

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: Epstein–Barr Virus (EBV), Human Papilloma Virus (HPV) and Mouse Mammary Tumor Virus (MMTV) are hormonal responsible viruses that re-emerges in cancer patients enhancing oncogenic potential. In the present study we investigated the presence of EBV, HPV and MMTV in inflammatory breast cancer (IBC) a rapidly progressing form of breast cancer characterized by specific molecular signature. We screened for anti-EBV IgG antibodies, anti-HPV IgG antibodies and anti-MMTV IgG antibodies in peripheral blood of 40 IBC invasive ductal carcinoma (IDC) and Lobular carcinoma. In addition, we screened for EBV, HPV-DNA and MMTV-mRNA. We also use sequence analysis to detect if we have a different virus strain or not. Our results reveal that IBC are characterized by astatically significant increase in EBV, HPV and MMTV IgG antibody titers and detection compared to healthy control patients. EBV, HPV- DNA and MMTV-mRNA was significantly detected in BC tissues than in than healthy control patients. EBV, HPV and MMTV sequence analysis not detect a different strains but by using phylogentic relationship it was homology with other strains in gen bank. The present study results demonstrated a correlation between EBV, HPV and MMTV and BC. Etiology , causality and transmission of EBV, HPV and MMTV infection with BC need more rigorously examined.
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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 (Glaser *et al.,* 2004). In Egypt, breast cancer ranked first among all tumors presented to the National Cancer Institute (NCI), Cairo, where it represents 17.5% of all diagnosed cancer cases (El Saghir *et al.,* 2007). Researches into breast cancer etiology are focused primarily on reproductive and other factors affecting circulating sex hormones and on genetic susceptibility. However, as identified risk factors are thought to explain the incidence in about half of all breast cancer cases only, researchers are motivated to consider other routes for disease pathogenesis (Madigan *et al.,* 1995; Mezzetti *et al.,* 1998; Martin and Weber, 2000; Hulka and Moorman, 2001). For many decades these and other oncogenic viruses have been hypothesised as having potential causal roles in breast cancer (Lawson and Heng, 2010). The main candidate viruses include EBV, high risk HPV and MMTV (Lawson and Heng, 2010).

A possible association of EBV with breast cancer was proposed as a consequence of the high incidence of male breast cancers, which was reported in Mediterranean countries, an area endemic for EBV; also, the occurrence of some EBV-associated lymphomas in the breast, and the morphological similarities between medullary carcinoma of the breast and nasopharyngeal carcinoma (NPC) (Mant *et al.,* 2004). Most of the studies performed to date to assess the association between EBV infection and breast carcinoma have been done in Western countries, however, consistent results have to be demonstrated from studies conducted all over the world in order to establish a causal relationship. So far, few studies have been reported from Asian countries (Joshi *et al.,* 2009; He *et al.,* 2011; Perrigoue *et al.,* 2012) and from Egypt (Bahnassy *et al.,* 2006; Mohamed *et al.,* 2007; Fawzy *et al.,* 2008).

Identification of the *Mouse mammary tumor virus* (MMTV) supports a viral etiology for breast tumors in animals, though similar germ line viral sequences found in humans are not believed to play any direct role in carcinogenesis (Serraino *et al.,* 2001). MMTV-like virus has been a major suspect as a cause of some human breast cancers for over 50 years. This is because MMTV is the well-established etiologic agent of mammary tumours in field and experimental mice and MMTV gene sequences have repeatedly been identified in human breast cancers

(Wang *et al.,* 1995; Lawson *et al.,* 2010; Mazzanti *et al.,* 2011).

It is accepted that *Human papillomavirus* (HPV) types 16 and 18 are carcinogenic, and that probably HPV types 31 and 33 are also carcinogenic in human cervical and anogenital cancers (IARC, 1995). The suspicion that HPVs may also have a role in human breast cancer is based on the identification of HPVs in human breast tumours and the immortalisation of normal human breast cells by HPV 16 and 18 (Band *et al,* 1990; De Villiers *et al,* 2005). *Human papilloma virus* 16 has been identified in breast tumours in Italian women and breast tumours in Norwegian women who had previous cervical neoplasia (Hennig *et al.,* 1999). No HPVs have been identified in normal breast tissues from normal women who have had cosmetic surgery. No significant correlations have been observed between HPVs identified in human breast cancer and hormone receptor status of the tumour. Recently, De Villiers *et al.* (2005) have identified a wide range of HPV types in cancer of the breast and nipples (De Villiers *et al.,* 2005).

The purpose of this study is to study molecular characterization of viruses involvement in breast cancer

2. 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 Geneal Sugery 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. Patients' profiles were obtained from medical history and demographical records including name, hospital name, ID number, age and sex. In addition, clinical data will include episode data, number and state of disease during episode and the empirical anti-viral, chemical or radio therapies given. Ten normal healthy women patients 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; very small tumours (< 1.0 cm) or tumours received in fixative were not included. Tumour 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 (10µl 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.

2.1. 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 Immuno diagnostics, Wurzburg, 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

2.2. 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% sodiumdodecylsulphate in 10mM Tris, 10mM EDTA, 50 mM NaC1) at 37°C overnight.

2.2.1. 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 10X*Taq* polymerase buffer (100mMTris-HCl, pH 8.8, 500mMKCl, 15mM $MgCl_2$, and 1% Triton X-100), 200µmol/l of each deoxynucleotide, 25pmol of EBV primers (5'-CACTTTAGAGCTCTGGAGGA-3' and 5'-TAAAGATAGCAGCAGCGCAG-3'), and1.25 units of *Taq* polymerase in a final volume of 50µl.After initial incubation for three minutesat 94°C, 40 PCR cycles were performed whichinvolved denaturing at 94°C for one minute,annealing at 57°C for 0.5 minute, and extension at72°C for 1.5 minute. The amplified product was 153 base pair fragment of the

Bam Hl "W" region of the the EBV *Bam*HI W internal repetitive fragment.

2.2.2. 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-bpfragment from the L1 regions of HPV-16 and 18 (D'Costa *et al.,* 1998). The final 50µl PCR mixture contained 10µl sample, 25µl PCR Master Mix (Promega), 3mM $MgCl_2$, 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.

2.2.3. PCR for MMTV

PCR was carried out using primers 2N(5'-CCTACATCTGCCTGTGTTAC-3') and 3N (5'-ATCTGTGGCATACCTAAAGG-3') which amplify an approximately 255-bpsegment from the MMTV *env* gene (Wang *et al.,* 1995). The amplification reaction was carried out with 100 pmol of primers using PCR BEADS from Amersham Pharmacia Biotech(Buckinghamshire, United Kingdom). Each reaction contained in 25ml, 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 $MgCl_2$; and (e) 200mM of each of the four nucleotides. Thermo cycling 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.

2.2.4. 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) minigels in 0.5X TBE buffer (Tris-borate-EDTA, 90mMTris acetate, 90mM boric acid, 2mM EDTA, pH 8.0) using 100 bp DNA ladder (Promega, Cat # G2101). The gels were visualized with UV light after staining for 10 min with 10µg/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 ~300 bp, ~371 bp and ~371 bpfor HPV, MMTV and EBV, respectively.

2.2.5.Cloning of PCR-amplified HPV, MMTV and EBV

HPV, MMTV and EBV cloning were carried out to clone the viruses genome using pGEM®-T Easy Vector System I (Promega, Cat # A1360). The recombinant plasmids were introduced into *E. coli* strain DH5α as described by manufacturer's instructions.

2.3. Sequence alignments and phylogenetic analyses

Multiple alignment of sequences was performed using DNAMAN software (Wisconsin, Madison, USA) and clustalw (Ver.1.74) program (Thompson *et al.,* 1994). The nucleotide distances were estimated considering alignment gaps and using the Jukes and Cantor's method (Jukes and Cantor, 1969) for correction of superimposed substitutions with the Molecular Evolutionary Genetics Analysis (MEGA) software (Ver. 4.0) (Tamura *et al.,* 2007). Phylogenetic relationships were evaluated using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) through DNAMAN software and Neighbour Joining (NJ) implemented through MEGA 4.0 software, and bootstrap analysis (1000 replicates) was performed to assess the reliability of the constructed phylogenetic tree.

3. Results and Discussion

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. All Cancer tissues (n=40) were screened for the presence of three different viruses using ELISA and PCR analysis. Breast cancer induced due to infection with viral agents namely, *Human papiloma virus* (HPV), *Epstein–Barr virus* (EBV) and *Mouse mammary tumor virus* (MMTV) were tested using ELISA.

3.1. Screening of viruses involvement in breast cancer by ELISA

Single virus in the same breast cancer specimen was identified frequently. ELISA for IgG antibodies against *Epstein–Barr virus* nuclear antigen (EBNA-1) was performed on serum samples of cases and control. Using manufacturer determined cut-off of 3 IU/ml, 0/40 cases (0%) and 1/10 controls (10%) were sero-positive for anti-EBNA-1 IgG (Tables 1, 2 & 7). ELISA for IgG antibodies against HPV16 L-1 was performed on serum samples of cases and control. Using manufacturer determined cut-off of 1 IU/ml, 6/40 cases (15%) in samples no. (22, 26, 30, 31, 33 and 34) and non-detected on controls for anti-HPV16 L-1 IgG (Tables 3, 4 & 7). ELISA for IgG

antibodies against MMTV WNT-1 was performed on serum samples of cases and control. Using manufacturer determined cut-off of 1ng/ml, 4/40 cases (10%) in samples no. 15, 16, 17, 20 and non-detected on controls for anti-MMTV WNT-1 (Tables 5, 6 & 7).

On the other hand, multiple viruses in the same breast cancer specimen were identified frequently. The prevalence of both EBV and HPV in the same specimen was (10/40) cases (25%) in samples no. (23, 24, 27, 28, 29, 32, 36, 37, 39 and 40), both of EBV and MMTV was (14/40) cases (35%) in samples no. 1, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 18, 21, HPV and MMTV together was no viruses. While all three viruses in same specimens was (5/40) cases (12.5%) in samples no. (2, 19, 25, 35 and 38). Only one patient sample no five not give any reaction with any of three selected viruses screening by ELISA., significantly in breast cancer specimens than normal control breast specimens (Table 7).

3.2. Screening of DNA extracted from 40 fresh frozen breast cancer specimens

Standard PCR was conducted at least 2 times on each sample of extracted DNA. DNA extracts from paraffin sections of all cases were subjected to PCR using primers (5'-CACTTTAGAGCTCTGGAGGA- 3') and (5'-TAAAGATAGCAGCAGCACAG-3') specific for the *Bam*HI W internal repetitive fragment of the EBV genome. After 35 amplification cycles, a PCR product of the expected size ~153 base fragment of DNA (Fig. 1) was observed in twenty seven of 40 cases (67.5%) as a single infection or mix with the other viruses. Indeed, EBV has been identified most consistently using PCR as in studies done by Horiuchi *et al.* (1994), Labrecque *et al.* (2001), Luqmani and Shousha (1995), Bonnet *et al.* (1999), Fina *et al.* (2001), Preciado *et al.* (2005), Tsai *et al.* (2005) and Perkins *et al.* (2006) with respective frequencies 66%, 20%, 40%, 51%, 31.8%, 35%, 45.2% and 46%.

Human papilloma virus DNA prepared from breast cancer tissues was amplified by PCR using degenerated primers MY09 (5'CGTCCMARRGGAWACTGATC-3') and MY11 (5'GCMCAGGGWCATAAYAATGG 3') which amplify ~452 base fragment of DNA. After amplification, the PCR products were analyzed using 1.5% agarose gel by electrophoresis and the results were ~452 base fragment of DNA (Fig. 2). Twenty of 40 cases (50%) were infected with HPV as a single infection or mix with the other viruses. Indeed, HPV has been identified most consistently using PCR as in studies done by Li *et al.* (2002), Widschwendter *et al.* (2004), Kan *et al.* (2005), Akil *et al.* (2008), He *et al.* (2009), Aceto *et al.* (2010) and Antonsson *et al.* (2011) with respective frequencies 68%, 64%, 48%, 61%, 60%, 60% and 50%.

Mouse mammary tumor virus DNA prepared from breast cancer tissues was amplified by PCR using primers (5'-CCTACATCTGCCTGTGTTAC-3') and (5'-ATCTGTGGCATACCTAAAGG-3') which amplify ~255 base fragment of DNA. After amplification, the PCR products were analyzed using 1.5% agarose gel by electrophoresis and the results were ~255 base fragment of DNA (Fig. 3). Twenty three of 40 cases were infected with MMTV as a single infection or mix with the other viruses.

In summary, HPV was detected in (7/40) cases 17.5% in samples no. (22, 26, 30, 31, 33, 34 and 39) and MMTV in (5/40) (12.5%) in samples no (15,16,17,19 and 20), of the 40 fresh frozen invasive breast cancer specimens tested by standard PCR while EBV 0/40 cases (0%) as single infection., While in control samples "Healthy Patients" it was detected only in EBV sample 1/10 cases (10%).The proportion of specimens in which no viruses were identified is significantly lower in breast cancer specimens than normal breast specimens (Table 8).

Multiple viruses in the same breast cancer specimen were identified frequently. The prevalence of both EBV and HPV in the same specimen was (9/40) cases (22.5%) in samples no. (23, 24, 27, 28, 29, 32, 36, 37 and 40), both of EBV and MMTV was (14/40) cases 35% in samples no. (1, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 18 and 21), HPV and MMTV together was no viruses While all three viruses in same specimens was (4/40) cases 10% in samples no (2, 25, 35 and 38) significantly in breast cancer specimens than normal control breast specimens (Table 8).

Glenn *et al.* (2012) conclude that (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. These three viruses may each have oncogenic roles in human breast cancer.

3.3. Screening of DNA in 10 normal controls

Standard PCR was done for fibrocystic disease of breast as a control group showed only detection for one case (1/10) (10%) for EBV only while the HPV 0 of 10 (0%) and MMTV 0 of 10 (0%) was not detected in all fibrocystic disease of breast as a control group (Table 8).

Table 1. Detection of EBNA-1 IgG by ELISA IBC Patients

Serial	EBNA IgG (U/ml)	Interpretation	Serial	EBNA IgG (U/ml)	Interpretation	Serial	EBNA IgG (U/ml)	Interpretation	Serial	EBNA IgG (U/ml)	Interpretation
1	13	Post.	11	14	Post.	21	16	Post.	31	3	Neg.
2	15	Post.	12	19	Post.	22	4	Neg.	32	18	Post.
3	13	Post.	13	15	Post.	23	16	Post.	33	8	Neg.
4	15	Post.	14	13	Post.	24	19	Post.	34	4	Neg.
5	8	Neg.	15	7	Neg.	25	13	Post.	35	20	Post.
6	16	Post.	16	8	Neg.	26	7	Neg.	36	13	Post.
7	14	Post.	17	10	Neg.	27	18	Post.	37	17	Post.
8	18	Post.	18	15	Post.	28	18	Post.	38	13	Post.
9	15	Post.	19	13	Post.	29	20	Post.	39	17	Post
10	17	Post.	20	5	Neg.	30	7	Neg.	40	14	Post.

Table 2. Detection of EBNA-1 IgG by ELISA in Control Group

Serial	EBNA IgG (U/ml)	Interpretation	Serial	EBNA IgG (U/ml)	Interpretation
1	4	Neg	6	8	Neg
2	6	Neg	7	6	Neg
3	2	Neg	8	5	Neg
4	4	Neg	9	2	Neg
5	13	Post.	10	1	Neg

Table 3. Detection of HPV16 L-1 IgG by ELISA in IBC Patients

Serial	HPV16 L-1IgG (U/ml)	Interpretation	Serial	HPV16 L-1IgG (U/ml)	Interpretation	Serial	HPV16 L-1IgG (U/ml)	Interpretation	Serial	HPV16 L-1IgG (U/ml)	Interpretation
1	0.409	Neg	11	0.214	Neg	21	0.282	Neg	31	.712	Post
2	0.625	Post	12	0.415	Neg	22	0.520	Post	32	0.641	Post
3	0.417	Neg	13	0.395	Neg	23	0.733	Post	33	0.574	Post
4	0.389	Neg	14	0.381	Neg	24	0.672	Post	34	0.598	Post
5	0.189	Neg	15	0.358	Neg	25	0.625	Post	35	0.852	Post
6	0.299	Neg	16	0.324	Neg	26	0.588	Post	36	0.753	Post
7	0.142	Neg	17	0.426	Neg	27	0.759	Post	37	0.535	Post
8	0.368	Neg	18	0.219	Neg	28	0.801	Post	38	0.836	Post
9	0.274	Neg	19	0.511	Post	29	0.528	Post	39	0.588	Post
10	0.235	Neg	20	0.168	Neg	30	0.612	Post	40	0.819	Post

Table (4): Detection of HPV16 L-1 IgG by ELISA in Control Group

Serial	HPV16 L-1 IgG (U/ml)	Interpretation	Serial	HPV16 L-1 IgG (U/ml)	Interpretation
1	0.158	Neg	6	0.123	Neg
2	0.312	Neg	7	0.318	Neg
3	0.224	Neg	8	0.254	Neg
4	0.218	Neg	9	0.098	Neg
5	0.154	Neg	10	0.128	Neg

Table 5. Detection of *MMTV WNT-1 IgG* by ELISA in IBC Patients

Serial	MMTV WNT-1IgG (ng/ml)	Interpretation	Serial	MMTV WNT-1IgG (ng/ml)	Interpretation	Serial	MMTV WNT-1IgG (ng/ml)	Interpretation	Serial	MMTV WNT-1IgG (ng/ml)	Interpretation
1	5	Post.	11	4.8	Post.	21	6.3	Post	31	0.54	Neg
2	8	Post.	12	2.7	Post.	22	0.24	Neg	32	0.24	Neg
3	4	Post.	13	3.8	Post.	23	0.6	Neg	33	0.3	Neg
4	5	Post.	14	7.6	Post.	24	0.5	Neg	34	0.6	Neg
5	0.35	Neg	15	1.3	Post.	25	0.8	Post	35	9	Post.
6	3	Post.	16	2	Post.	26	0.2	Neg	36	0.48	Neg
7	2.4	Post.	17	1.1	Post.	27	0.2	Neg	37	0.23	Neg
8	3	Post.	18	2.2	Post.	28	0.4	Neg	38	8	Post.
9	4.7	Post.	19	1.11	Post.	29	0.32	Neg	39	0.4	Neg
10	3.9	Post.	20	1.9	Post.	30	0.47	Neg	40	0.79	Neg

Table 6. Detection of MMTV WNT-1 IgG by ELISA in Control Group

Serial	WNT-1 (ng/ml)	Interpretation	Serial	WNT-1 (ng/ml)	Interpretation
1	0.158	Neg	6	0.123	Neg
2	0.312	Neg	7	0.318	Neg
3	0.224	Neg	8	0.254	Neg
4	0.218	Neg	9	0.098	Neg
5	0.154	Neg	10	0.128	Neg

Table 7. 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 ELISA

	EBV	HPV	MMTV	EBV + HPV	EBV + MMTV	HPV + MMTV	EBV + HPV + MMTV	NO Virus
Breast Cancer (n=40)	0(0%)	6(15%)	4(10%)	10(25%)	14(35%)	0(0%)	5(12.5%)	1(2.5%)
Normal breast (n=10)	1(10%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(10%)

Table 8. Screening of viruses involvement in breast cancer by PCR

Serial	Detection of **(EBV)** by PCR	Detection of **(HPV)** by PCR	Detection of **(MMTV)** by PCR
1	Post	Neg.	Post
2	Post	Post	Post
3	Post	Neg.	Post
4	Post	Neg.	Post
5	Neg.	Neg.	Neg.
6	Post	Neg.	Post
7	Post	Neg.	Post
8	Post	Neg.	Post
9	Post	Neg.	Post
10	Post	Neg.	Post
11	Post	Neg.	Post
12	Post	Neg.	Post
13	Post	Neg.	Post
14	Post	Neg.	Post
15	Neg.	Neg.	Post
16	Neg.	Neg.	Post
17	Neg.	Neg.	Post
18	Post	Neg.	Post
19	Neg.	Neg.	Post
20	Neg.	Neg.	Post
21	Post	Neg.	Post
22	Neg.	Post	Neg.
23	Post	Post	Neg.
24	Post	Post	Neg..
25	Post	Post	Post
26	Neg.	Post	Neg.
27	Post	Post	Neg.
28	Post	Post	Neg.
29	Post	Post	Neg.
30	Neg.	Post	Neg.
31	Neg.	Post	Neg.
32	Post	Post	Neg.
33	Neg.	Post	Neg.
34	Neg.	Post	Neg.
35	Post	Post	Post.
36	Post	Post	Neg.
37	Post	Post	Neg.
38	Post	Post	Post.
39	Neg.	Post	Neg.
40	Post	Post	Neg.

Table 9. 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

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

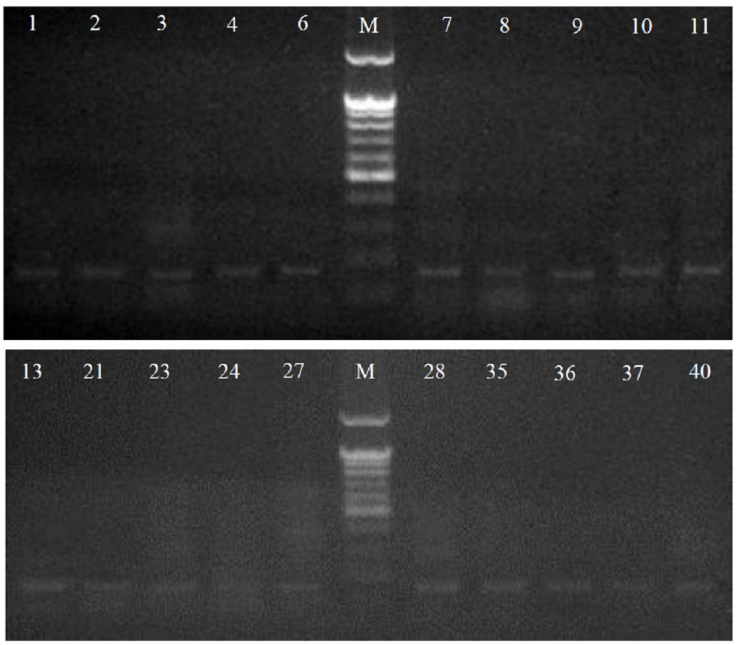

Figure 1. Electrophoresis of 1.5% agarose gel showing *Epstein–Barr virus* (EBV) screen of patient samples using specific primer. Lane M is DNA molecular weight marker (100 bp ladder).

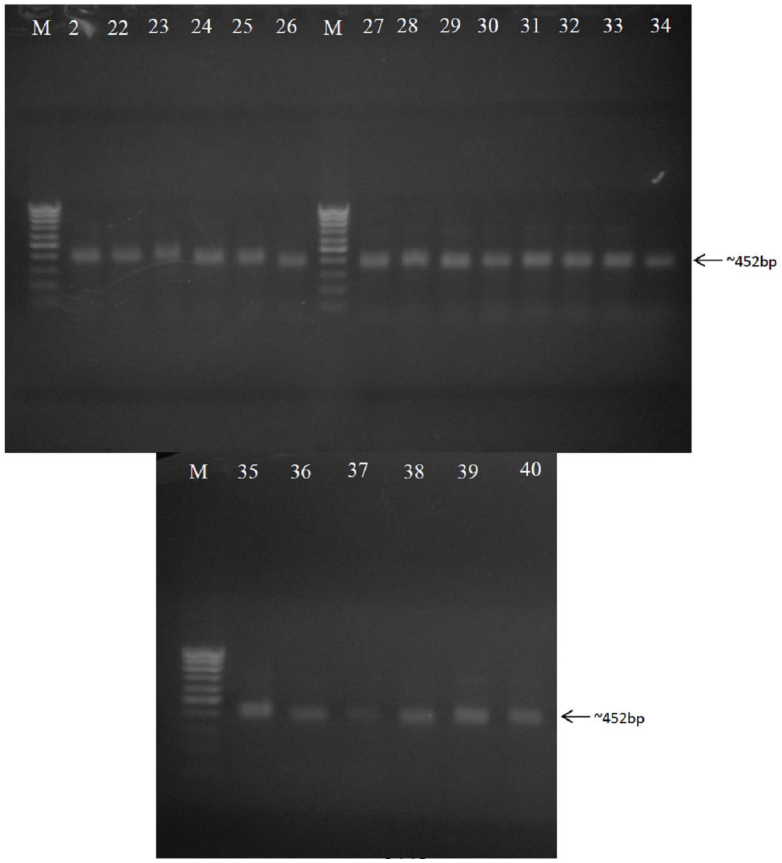

Figure 2. Electrophoresis of 1.5% agarose gel showing *Human papilloma virus* (HPV) screen of patient samples using MY primer. Lane M is DNA molecular weight marker (100 bp ladder).

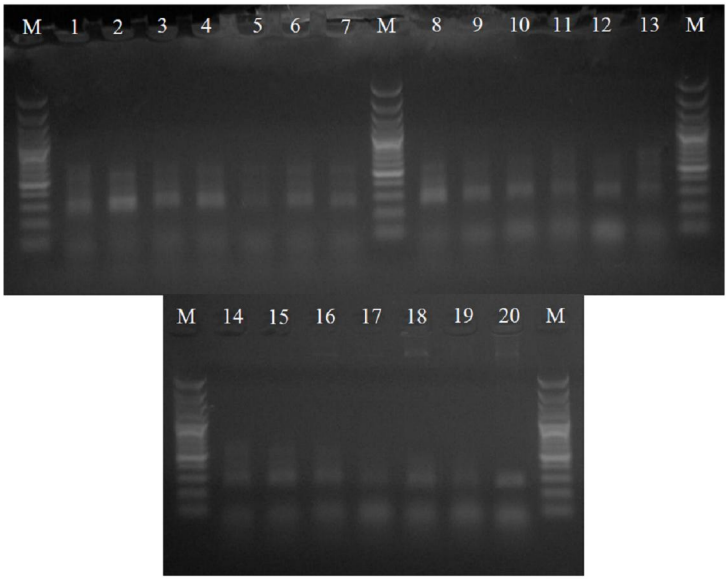

Figure 3. Electrophoresis of 1.5% agarose gel showing *Mouse mammary tumor virus* (MMTV) screen of patient samples using specific primer. Lane M is DNA molecular weight marker (100 bp ladder).

3.4. Statistical analysis of nucleotide sequence and alignment

3.4.1. *Bam*HI W internal repetitive fragment of the EBV

The partial nucleotide sequence of the *Bam*HI W internal repetitive fragment (135 nucleotides) of the EBV-EG strain revealed the highest content for Adenine (A) 39 (25.5%) and Cytosine (C) 39 (25.5%) followed byGuanine (G) 38 (24.8%), and Thymine (T) 37 (24.2%).Data also showed that, C+G content was 77 (50.3%) and A+T content was 76 (49.7%) the ratio between C+G to A+T was 1.01. The sequence was compared with similar sequences retrieved from DNA databases by using the NCBI n-BLAST search program in the National Center for Biotechnology Information (NCBI) and was multiple-aligned at the same partial sequences of 8 reported *Bam*HI W internal repetitive fragment of EBV sequences in GenBank using ClustalW program with minor manual adjustments, resulting in 135 positions including the gaps.

3.4.2. L1 gene of for *Human papilloma virus* Egyptian strain (HPV-Egypt)

The partial nucleotide sequence of L1 gene (452 nucleotides) for HPV-EG strain revealed the highest content for Adenine (A) 151 (33.4%) followed by Thymine (T) 135 (29.9%), then Cytosine (C) 89 (19.7%), and Guanine (G) 77 (17%). Data also showed that, the ratio between C+G to A+T was 0.58. The sequence was multiple-aligned at the same partial sequences of 9 reported HPV sequences in GenBank using ClustalW program with minor manual adjustments, resulting in 452 positions including the gaps. The amplified L1 gene sequence of isolate HPV-Egypt was most closely related to that of HPV isolates under GenBank accession numbers, AF472509, AF536180, JQ004098 and HQ644299 where showed 99.8% identity with these isolates. On the other hand, it was showed 99.6% identity with HPV isolates under GenBank accession numbers, HQ644281, JQ004095, HQ644257, HQ644240 and HQ644298. A phylogenetic tree was generated using the Neighbour-Joining method and bootstrap analysis of 1000 repetitions (Fig. 4). The amino acids sequence of L1 gene for isolate HPV-Egypt was produced 20 types of 150 amino acids beginning with Alanine (A) and ended with Glycine (G). Since the amino acids sequence was most closely related to that of HPV isolates under GenBank accession numbers, AF472509, JQ004098 and HQ644281 where showed 100% identity with these isolates. While it was showed 99.3% identity with HPV isolates under GenBank accession numbers, AF536180, HQ644299, JQ004095, HQ644257, HQ644240 and HQ644298 (Fig. 5).

Human papilloma viruses are mainly transmitted by cell surface contact. Therefore, how are HPVs transmitted to the breast? A suggestive clue comes from the observation by De Villiers *et al.* (2005) that HPVs are present in cancers occurring in human nipple milk ducts and that these cancers have the typical histological features of HPV-induced human cancers. In addition, in two independent studies, HPV 16 has been found to be present in breast tumours that occur in European women with

HPV 16-associated cervical cancer (Hennig *et al.,* 1999; Widschwendter *et al.,* 2004). Virions (virus particles) are shed from desquamating keratinocytes (the target cells for HPV infections) and high-risk HPVs can be transmitted by close human nonsexual contact (Bryan and Brown, 2001; Rintala *et al.,* 2005). Accordingly, HPVs may be transmitted by hand from the female perineum to the breast which could occur, for example, during showering or bathing (Kan *et al.,* 2005).

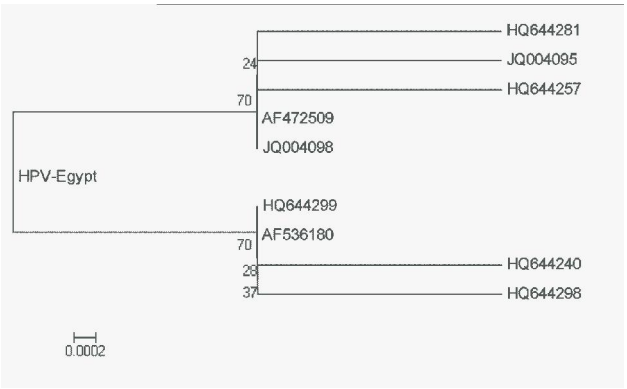

Figure 4. Neighbour-joining tree of nucleotide sequence of L1 gene for HPV-Egypt and the other 9 isolates of HPV published in GenBank. Numbers represent bootstrap percentage values based on 1000 replicates.

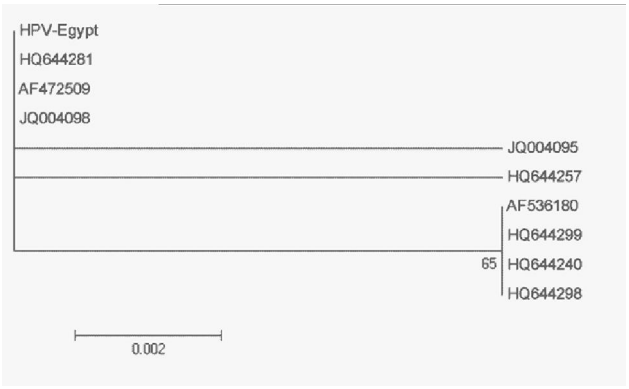

Figure 5. Neighbour-joining tree of amino acids sequence of L1 gene for HPV-Egypt and the other 9 isolates of HPV published in GenBank. Numbers represent bootstrap percentage values based on 1000 replicates.

3.4.3. Envelope polyproteingene of for *Mouse mammary tumor virus* Egyptian strain (MMTV-Egypt)

The partial nucleotide sequence of envelope polyprotein gene (255 nucleotides) for MMT-EG strain revealed the highest content for Thymine (T) 88 (34.5%) followed by Adenine (A) 65 (25.5%), then Cytosine (C) 54 (21.2%), and Guanine (G) 48 (18.8%). Data also showed that, the ratio between C+G to A+T was 0.67. The amplified envelope polyprotein gene sequence of isolate MMTV-Egypt was most closely related to that of MMTV isolates under GenBank accession numbers, AY702714, and AY702716 where showed 99.2% identity with these isolates. On the other hand, it was showed 98.8% identity with MMTV isolates under GenBank accession numbers, AY152721, AY702717, and JX843708. As well as, it was showed 98.4% identity with MMTV isolates under GenBank accession numbers, EF495356, AF228552, AY152722, AY702715, and AY702718. A phylogenetic tree was generated using the Neighbour-Joining method and bootstrap analysis of 1000 repetitions (Fig. 6). The amino acids sequence of partial envelope polyprotein gene for isolate MMTV-Egypt was produced 19 types of 85 amino acids beginning withProline (P) and ended with Aspartate (D). Since The amino acids sequence was most closely related to that of MMTV isolates under GenBank accession numbers, AY702714, and AY702716 where showed 98.8% identity with these isolates. On the other hand, it was showed 97.6% identity with MMTV isolates under GenBank accession numbers, AY152721, AY152722, AY702717, AY702718, EF495356, and JX843708. As well as, it was showed 96.5% identity with MMTV isolates under GenBank accession numbers, AY702715, and AF228552. A phylogenetic tree was generated using the Neighbour-Joining method and bootstrap analysis of 1000 repetitions (Fig. 7).

Wang *et al.* (1995) reported that unique MMTV env gene sequences are present in 38.5% of the BC samples analyzed and 39.7% of archival samples of BC, and that these sequences are absent in normal tisues, including lymphocytes from patients with positve BC and in cancers other than breast. Normal breast tisue and fibroadenomas have a low frequency (1.8-6.9%) of positive results. When cloned and sequenced, the sequences were found to be highly homologous to the MMTV env gene, but not to the endogenous retroviral sequences (Ono *et al.,* 1986).

4. 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

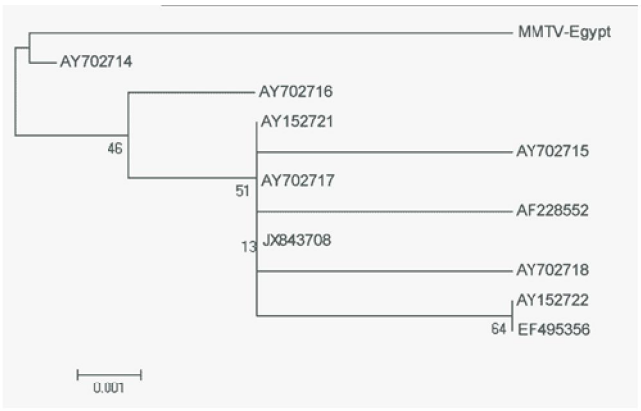

Figure 7. Neighbour-joining tree of amino acids sequence of envelope polyprotein gene for MMTV-Egypt and the other 10 isolates of MMTV published in GenBank. Numbers represent bootstrap percentage values based on 1000 sreplicates.

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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

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