Original Article

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Is HPV Virus Associated with Human Breast Cancer? Time to Re-examine the Postulate. Egyptian Study

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ABSTRACT

Background: There is increasing evidence that high-risk human papilloma virus (HPV) is involved in cancers in addition to cervical cancer. Infectious agents are thought to be responsible for approximately 16% of cancers worldwide, however there are mixed reports in the literature regarding the prevalence and potential pathogenicity of viruses in breast cancer.

Methods: We screened 30 fresh frozen breast cancer tissue specimens collected immediately postoperative from patients in Ain-Shams University Hospital for the presence of human papilloma virus (HPV) DNA by PCR and Koilocytic changes in the breast cancer tissue.

Results: Overall prevalence of HPV in malignant breast tissue was 16.7% In addition, we found that the oncogenic characteristics of HPV associated breast cancer are very similar to HPV-associated cervical cancer. Specifically, that putative koilocytes are present in some HPV associated breast cancers.

Conclusion: The above observations indicate a likely causal role for high-risk HPV in human breast cancer and offer the possible role of HPV in breast cancer prognosis.

Keywords: *Breast Cancer; Human Papillomavirus; Koilocytes; PCR*

Introduction

Breast cancer is the most common cancer affecting women worldwide. 1.7 million new cases were reported in 2012, representing about 12% of all new cancer cases and about 25% of all cancers in women. Although incidence trends for older women have recently stabilized, younger women are experiencing a rising incidence [1]. Many attempts showed the association of viruses such as mouse mammary tumour virus (MMTV), herpes viruses, cytomegalovirus and human papilloma virus (HPV) in malignant breast tissue [2]. There are 200 different HPV serotypes; a small number of these called "high-risk" types are associated with cancers [3]. A role for HPV in other cancers is now well accepted as a causal role in head and neck cancers [4].

Many studies showed the presence of HPV high-risk types 16, 18 and 33 in breast cancer specimens from different countries around the world: Italy, Norway, China, Japan, USA, Austria, Brazil, Australia, Taiwan, Turkey, Greece, Korea, Mexico, Hungary and Syria with prevelance rates ranging from 4% to 86% [5,6,7,8]. In all studies, high-risk HPV was found in tumour tissue only and not in surrounding normal tissue, except one study done in Turkey, in which the virus was also detected in normal tissue [9]. The HPV oncoproteins E6 and E7 which have been found to interact and inactivate the two principal host cell tumor suppressor proteins p53 and Rb respectively are also shown to immortalize human mammary epithelial cells in-vitro [10]. Although the route of transmission for the virus has not been determined, women positive for both breast and cervical cancers were found to be infected with the same HPV type in both tumours [11]. Koilocytes are large epithelial cells with a vacuolated halo surrounding a dense nucleus. They are specific and indicative of HPV infection being caused by the action of HPV E5 and E6 oncoprotiens [12].

Materials and Methods:

Sample Collection. All the patients participated in this study were diagnosed as breast cancer by clinical examination, sonomammography and ultrasound guided core biopsy at our breast unit- General surgery department- Ain shams university hospital. The study was conducted over a period of 4 months from January 2016 till April 2016. 30 malignant breast tissue specimens from patients with breast cancer were aseptically collected by one surgical team immediately after tumor resection. The sample was placed into a sterile tube and transported to the PCR Laboratory, snap frozen and stored at − 80 °C. The tumors were resected by modified radical mastectomy (MRM) in 18 cases and wide local excision with axillary clearance (WLE+Axcl) in 12 cases.

Demographic features including age, family history, menstrual history, imaging studies were collected from medical records. None of the patients had history of cancer cervix. Evaluation of pathologic features of the tumor including tumor size, tumor type according to WHO classification, tumor grade, lymph node metastasis, marginal involvement, nipple and areola involvement, TNM stage and hormonal status was done.

According to immunohistochemical profile and DNA (microarrays), breast tumors were classified into four groups [13, 14]:

- Luminal A: ER+, PR+, Her2 -
- Luminal B: ER+,PR+, Her2 +
- Triple negative: ER-, PR-, Her2 –
- Her2: ER-, PR -, Her2 +

DNA Extraction: First, Fresh frozen breast tissue were lysed with buffer ATL & proteinase K by adding 360 ul ATL + 40 ul proteinase K in a sterile 2ml tube incubate at 37 ^{O}C for extended period of time until tissue were completely lysed. Next, 400ul of buffer AL was added to the tube & incubate at 56 ^{O}C for 30 min until the mixture was very clear. Then, 400ul of ethanol was added, vortexed & whole solution was transferred in 2 steps to the Qiagen spin column and centrifuged at 8000 rpm for 1 min in each step & spin column was transferred to a new 2ml tube and was washed with Qiagen wash 1 & wash2 using 500ul of wash solution . Last, Spin column was transfer to a new elution tube &100ul of elution buffer was added to spin column and left to stand at room temperature for 5min, centrifuged at 6000 rpm for 1 min.

PCR analysis: We used (Qiagen, Dusseldorf, Germany) hot start ready to use master mix in a volume of 50 ul +primers MYO9(5′ -GCM CAG GGW CAT AAY AAT GG-3 ′) and MY11(5′ -CGT CCM ARR GGA WAC TGA TC-3′) , for the first run of PCR using the following cycling parameters by holding at 95 ^{O}C for 15 minutes followed by 40 cycles of 94 ^{O}C for 45sec , 55 ^{O}C for 45sec , 72 ^{O}C for 1min and final extension at 72 ^{O}C for 10 min. This was followed by nested PCR using 2ul of the first PCR product added to 48ul of master mix containing primers GP5+(5′ -TTT GTT ACT GTG GTA GAT ACT AC-3′) and GP6+(5′ -GAA AAA TAA ACT GTA AAT CAT ATT C-3′) with the following cycling parameters by holding at 95 ^{O}C for 15 min followed by 40 cycles of 94 ^{O}C for 1min , 40 ^{O}C for 1min , 72 ^{O}C for 1min and final extension at 72 ^{O}C for 10 min. These primers were degenerate for HPV16 and 18. In addition, tissues were screened b-globin to confirm their suitability for this study. After PCR, detection of amplified product was done by agarose gel electrophoresis to detect amplified product at 150 base pair in positive cases.

Detection of koilocytic changes by Histopathology: The presence of koilocytosis in the fixed breast cancer series was assessed by light microscopy with koilocyte positive cervical cancer specimens used for comparison. Koilocytes were best characterized by the presence of large cells with relatively small, but irregular and hyperchromatic nuclei surrounded by clear and transparent cytoplasm, as shown in figures (1 to 5). Koilocytosis is restricted to the replicating basal cells and multinucleation is common in these cells [15].

Results

Clinical and pathological characterization of patients who participated in the present study is described in Table 1.

Application of Multiplex PCR for detection of HPV in breast cancer tissue revealed 5 (16.7%) out of 30 patients positive for HPV as shown in graph (1) and figure (6).

Regarding the Clinical and pathological characterization in correlation with the PCR results shown in table 2.

There was a strong correlation with highly significant values regarding the age (p value=0.000), T staging (p value=0.002) as shown in graph (2), her 2 neu (p value=0.000), Koilocytic changes (p value =0.00001) and the PCR results for Human Papilloma Virus as shown in graph (3). While there was significant statistical values regarding the margins (p value =0.023), N staging (p value= 0.046) as shown in graph (4), size (p value=0.038) and the PCR results for Human Papilloma Virus.

Table 1: Demographic features of patients and Clinical and pathological characterization of the tumors.

Age	**Mean±SD**	**53.77±11.00**
	Range	**38–84**
Marital Status	married	23(76.7%)
	single	7(23.3%)
TLC	Mean±SD	7.06±1.54
	Range	5–9.4
Neutrophils	Mean±SD	56.30±8.49
	Range	46–71
Lymphocytes	Mean±SD	33.90±7.93
	Range	18–45

Age	Mean±SD	53.77±11.00
	Range	38–84
Monocytes	Mean±SD	6.07±1.76
	Range	3–9
Eosinophils	Mean±SD	2.43±0.82
	Range	0–4
Heamoglobin	Mean±SD	12.64±1.15
	Range	11–14.4
Platelets	Mean±SD	334.60±55.33
	Range	248–424
Ultra sound	BIRAD III	2(6.7%)
	BIRAD IV	4(13.3%)
	BIRAD V	20(66.7%)
	BIRAD VI	4(13.3%)
Site	bilateral	4(13.3%)
	left	14(46.7%)
	right	12(40.0%)
Size	Mean±SD	3.76±1.59
	Range	2–7
Nipple/skin affection	No	25(83.3%)
	Yes	5(16.7%)
Tumor histologic type	IDC ILC DCIS	16 (53.3%) 8 (26.7%) 6 (20%)
T stage	T1	1(3.3%)
	T2	22(73.3%)
	T3	5(16.7%)
	T4	2(6.7%)
Focality	multifocal	10(33.3%)
	unifocal	20(66.7%)
Surgery type	MRM	18(60%)
	WLE+ Axcl	12(40%)
Grade	II	24(80.0%)
	III	6(20.0%)
Lumina A & Luminal B	No	8(26.7%)
	Yes	22(73.3%)
Her2 neu	0	24(80.0%)
	1	2(6.7%)
	2	2(6.7%)
	3	2(6.7%)
Triple negative	No	28(93.3%)
	Yes	2(6.7%)
N stage	N0	12(40.0%)
	N1	9(30.0%)
	N2	3(10.0%)
	N3	6(20.0%)
Koilocytic changes	Negative	20(66.7%)
	Positive	10(33.3%)
PCR	Negative	25(83.3%)
	Positive	5(16.7%)

IDC: Invasive duct carcinoma, ILC: Invasive lobular carcinoma, DCIS: Duct carcinoma in situ, HP: Hormone positive, MRM: Modified radical mastectomy, WLE+Axcl: Wide local excision with axillary clearance, BIRADS: Breast Imaging Reporting and Data System.

Table 2: Clinical and pathological characterization in correlation with the PCR results

		Negative PCR	Positive PCR	Chi-square test	
		n = 25	n = 5	X^2	P-value
Age	Mean±SD	50.76±6.82	68.80 ± 16.10	-4.201	0.000 **HS***
	Range	38–63	52 – 84		
Marital Status	married	20(80.0%)	3 (60.0%)	0.932	0.334 **NS**
	single	5(20.0%)	2 (40.0%)		
Neutrophils	Mean±SD	57.16±8.92	52.00 ± 4.18	1.253	0.220 **NS**
	Range	46–71	48 – 58		
Lymphocytes	Mean±SD	32.84±8.01	39.20 ± 5.36	-1.688	0.103 **NS**
	Range	18–44	34 – 45		
Monocytes	Mean±SD	6.08±1.78	6.00 ± 1.87	0.091	0.928 **NS**
	Range	3–9	4 – 8		
Eosinophils	Mean±SD	2.44±0.71	2.40 ± 1.34	0.098	0.922 **NS**
	Range	2–4	0 – 3		
Hemoglobin	Mean±SD	12.66±1.08	12.56 ± 1.60	0.168	0.868 **NS**
	Range	11.2–14.4	11 – 14.2		
Platelet	Mean±SD	341.24±52.96	301.40 ± 60.91	1.501	0.144 **NS**
	Range	248–424	260 – 407		
site	bilateral	4 (16.0%)	0 (0.0%)	2.829	0.243 **NS**
	left	10 (40.0%)	4 (80.0%)		
	right	11 (44.0%)	1 (20.0%)		
Size	Mean±SD	3.50 ± 1.48	5.10 ± 1.64	-2.183	0.038 NS
	Range	2 – 7	2.5 – 6.5		
nipple/skin	No	22 (88.0%)	3 (60.0%)	2.352	0.125 **NS**
	Yes	3 (12.0%)	2 (40.0%)		
Tumour type	IDC ILC DCIS	12 (48%) 8 (32%) 5 (20%)	4 (80%) 0 1 (20%)	2.4	0.301 **NS**
T stage	T1	1 (4.0%)	0 (0.0%)	14.487	0.002 **HS***
	T2	21 (84.0%)	1 (20.0%)		
	T3	3 (12.0%)	2 (40.0%)		
	T4	0 (0.0%)	2 (40.0%)		
Focality	Multifocal	10 (40.0%)	0 (0.0%)	3.000	0.083 **NS**
	Unifocal	15 (60.0%)	5 (100.0%)		
Grade	II	19 (76.0%)	5 (100.0%)	1.500	0.221 **NS**
	III	6 (24.0%)	0 (0.0%)		
luminal A & luminal B	0	6 (24.0%)	2 (40.0%)	0.545	0.460 **NS**
	1	19 (76.0%)	3 (60.0%)		
her2	negative	23 (92.0%)	1 (20.0%)	23.100	0.000 **HS***
	1	2 (8.0%)	0 (0.0%)		
	2	0 (0.0%)	2 (40.0%)		
	3	0 (0.0%)	2 (40.0%)		
Triple negative	0	23 (92.0%)	5 (100.0%)	0.429	0.513 **NS**
	1	2 (8.0%)	0 (0.0%)		
N stage	N0	12 (48.0%)	0 (0.0%)	8.000	0.046 **S**
	N1	5 (20.0%)	4 (80.0%)		
	N2	3 (12.0%)	0 (0.0%)		
	N3	5 (20.0%)	1 (20.0%)		
Koilocytic changes	Negative	23 (92%)	0 (0.0%)	19.7143	0.00001 **HS***
	positive	2 (8%)	5 (100.0%)		

IDC: Invasive duct carcinoma, ILC: Invasive lobular carcinoma, DCIS: Duct carcinoma in situ, HP: Hormone positive, MRM: Modified radical mastectomy, WLE: Wide local excision , BIRADS: Breast Imaging Reporting and Data System .

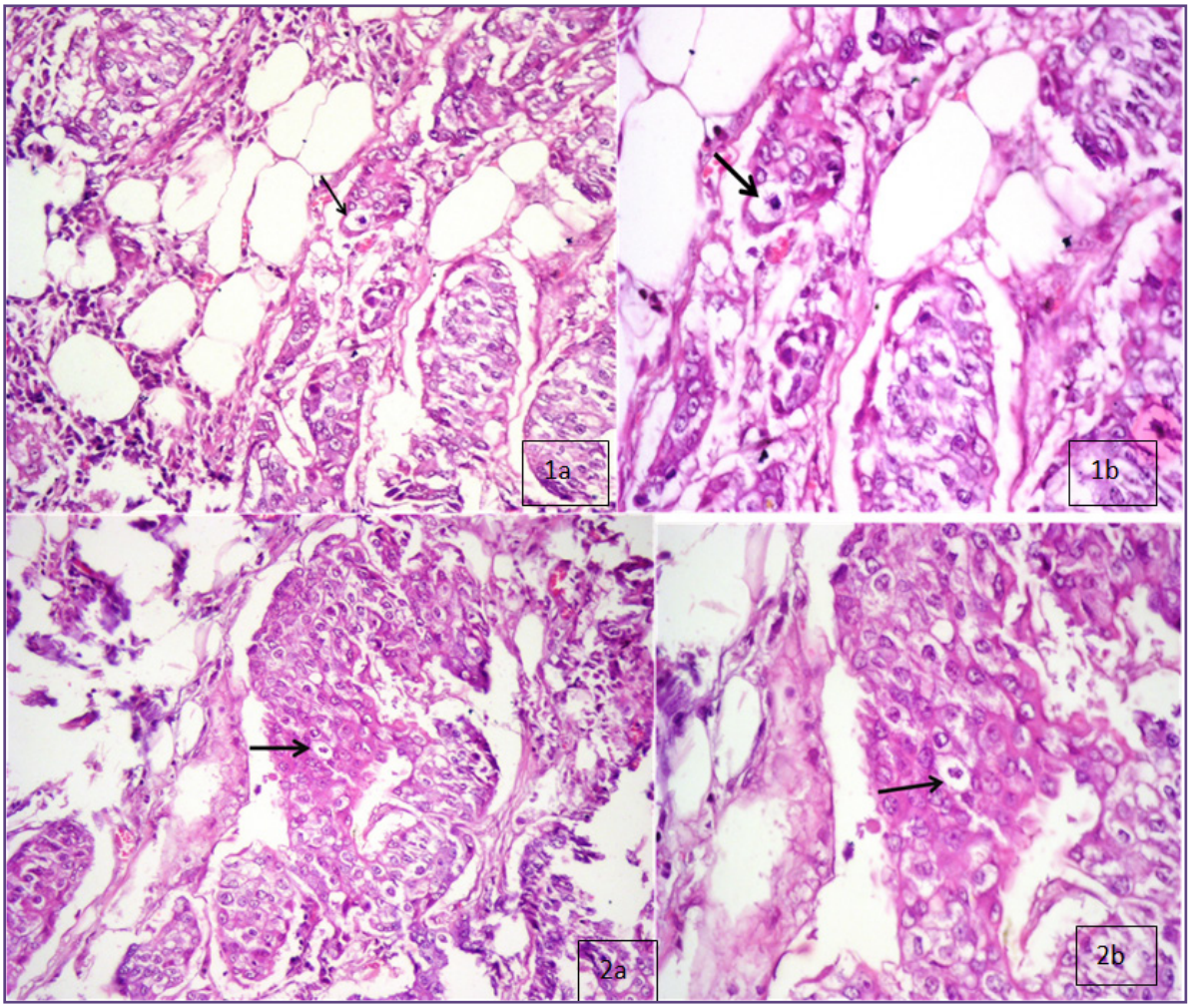

Fig. 1a, 1b and 2a, 2b: two cases of invasive duct carcinoma showing epithelial cells with koilocytic changes (arrows) in the form of clear cytoplasm and condensed nucleus (H&E; original magnification: 1a and 2a x200, 1b and 2b x400).

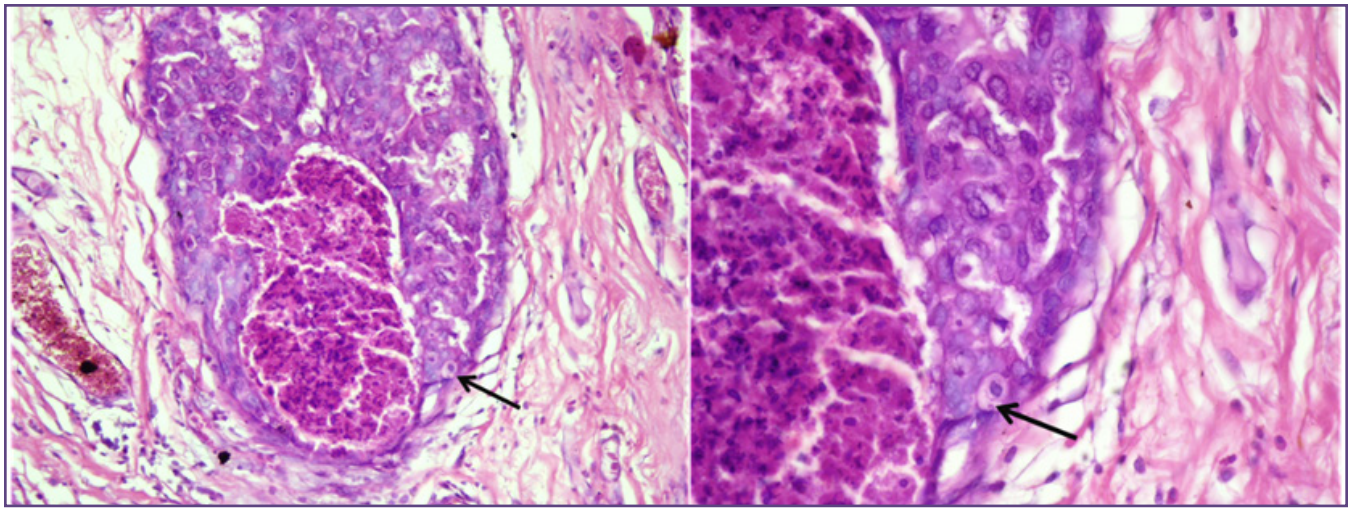

Fig. 3a, 3b: Duct carcinoma in situ; comedo type with occasional koilocytic changes (arrows) (H&E; original magnification: 3ax200, 3bx400).

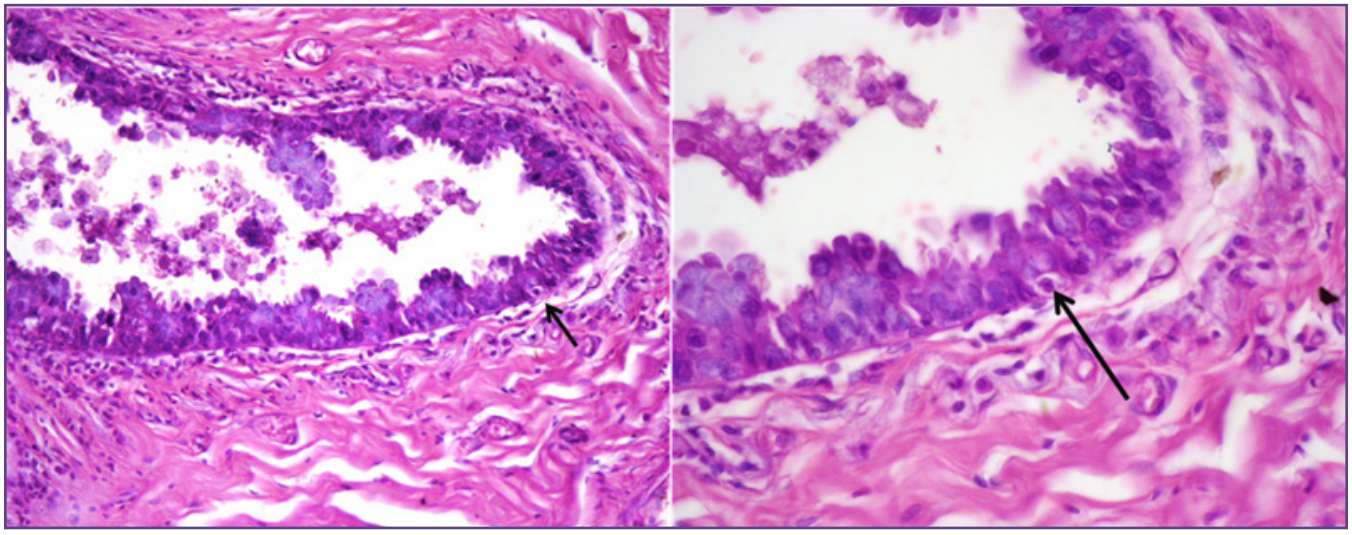

Fig. 4a, 4b: Duct carcinoma in situ; micropappillary type with occasional koilocytic changes at the basal layer (arrows) (H&E; original magnification: 4ax200, 4bx400).

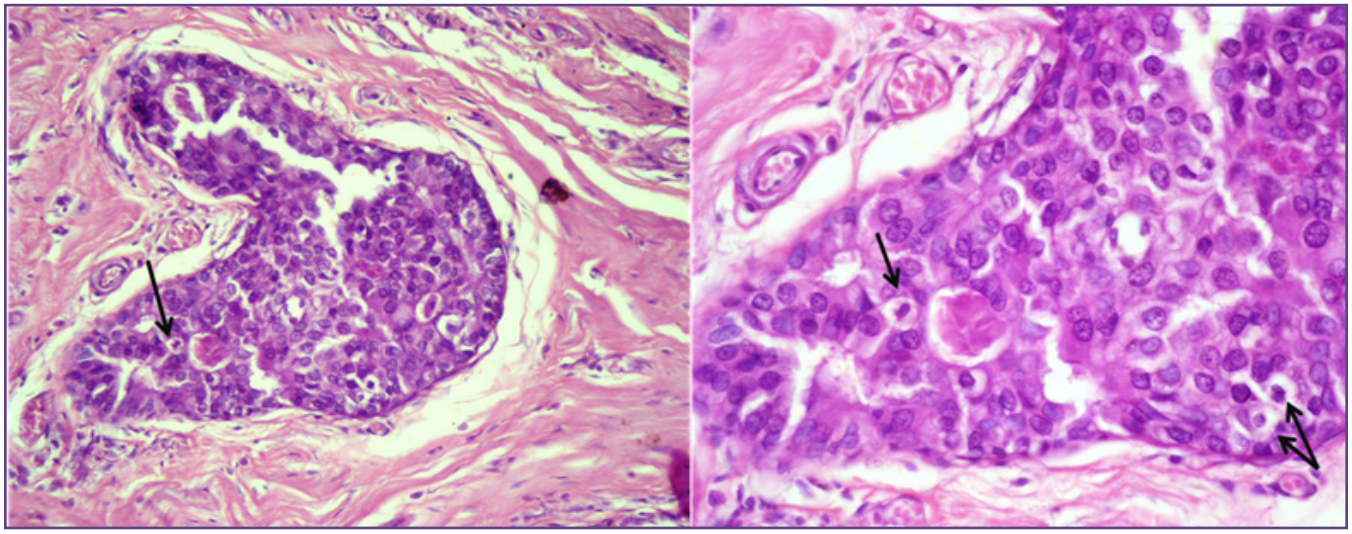

Fig. 5a, 5b: Duct carcinoma in situ; cribriform type with occasional koilocytic changes (arrows) (H&E; original magnification: 5ax200, 5bx400).

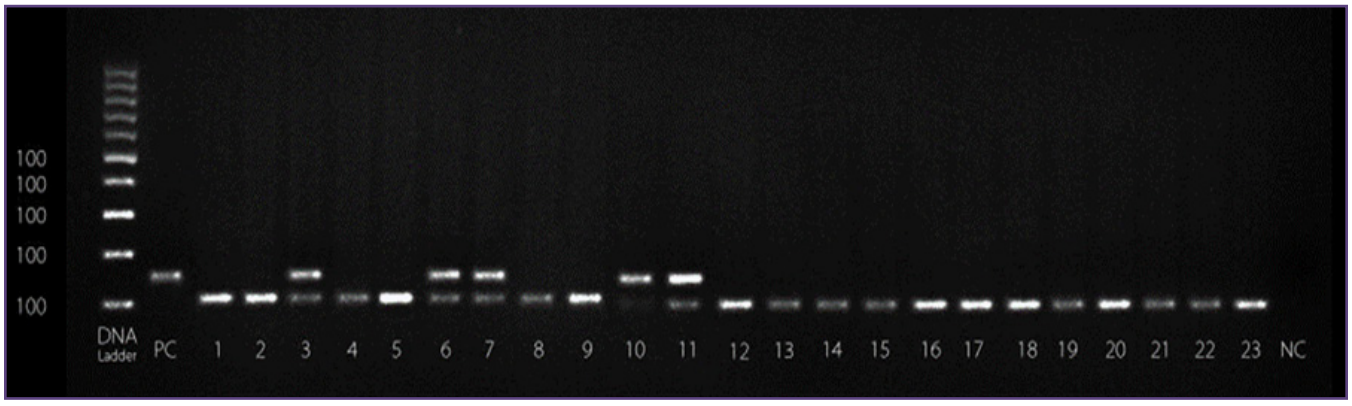

Fig. 6: Gel electrophoresis showing positive samples number (3, 6,7,10 and 11).

Discussion

HPV infection was diagnosed in 16.7% of breast carcinoma cases, involving 33.3% of Invasive Ductal Carcinoma cases and 16.7% of Duct Carcinoma in situ cases included in this study, while all cases of Invasive lobular Carcinoma were negative for HPV infection.

We used multiplex PCR in association with routine H&E examination for koilocytic changes to confirm the presence of HPV infection. Another Egyptian study reported HPV infection in 24.3% of invasive breast carcinoma cases, but they used immunohistochemistry in their study [16]. Many studies have reported variable prevalence of HPV infection in breast carcinoma cases. Our results were comparable to an Australian study [15] which identified high-risk HPV DNA sequences in the nuclei of breast cancer epithelial cells in (39%) of duct carcinoma in situ and (23%) of invasive duct carcinoma breast cancer specimens. The noticeable lower percentage of HPV involvement in Duct Carcinoma in situ in our series may be attributed to lower number of cases and sampling techniques. On the other hand a Chinese study from Hong Kong reported negative HPV sequences in all blood and breast tissues by real time PCR performed on 102 cases of breast carcinomas [17]. Also Lindel etal. [18] conducted a study on 81 Swiss breast cancer females who were negative for HPV DNA by PCR and stated that the role of HPV in etiology of breast carcinoma was not supported . These discrepancies in prevalence indicate that ethnic differences play a role in susceptibility to HPV infection and consequently associated breast cancer. Other factors include variability in sample size, differences in immunological and molecular methods, and inter-laboratory variability in sample collection and handling. Also false positive results may be acquired by PCR because it can't indicate which type of cells the virus has infected, as HPV was reported to be present in normal breast tissue as well or contamination while handling the sample may be a possible cause [18]. In our study we tried to minimize these errors by combining the use of H&E sections for detection of koilocytosis in association with PCR results to increase the accuracy of diagnosis. We didn't use immunohistochemistry for HPV because of its low sensitivity, as proved P. DeVilliers etal.[19] who reported negative immunohistochemical expression of all high risk HPV cases that were positive for PCR , yet other studies demonstrated positive P16 in cases negative for HPV [20].

Regarding the Clinical and pathological characterization in correlation with the PCR results in this study; there was a highly statistically significant correlation between HPV and the mean for age. The patients with proved HPV infection had a significantly older age than those without HPV infection. These results are contrary to Girianelli et al [21] and Akarolo-Anthony et al [22] who observed a reduction in high-risk HPV prevalence with aging.

Regarding the relationship between HPV and prognostic factors of breast cancer, we detected a statistically significant correlation between HPV positive cases and tumor size *(P= 0.38),* lymph node metastasis (N stage) (*p=0.046*) and tumor stage (T stage) *(P=0.002);* all the cases in our study presented by T4 were positive for HPV. We assume that HPV doesn't only play a role in etiology of breast carcinoma, but is implicated also in tumor progression and spread. The link between HPV and tumor metastasis was be explained by Yasmeen et al. [23] and Akil et al.[24] who detected an association between high risk HPV and the upregulated expression of Id-1 transcription factor in aggressive breast cancer tissues and suggested that the virus can induce cell invasion and metastasis via Id-1. Also, Cavuslu et al. [25] supported our hypothesis by proving that HPV was predictive of metastasis in their series of patients with cervical carcinoma. Contrary to our results, Fernandes et al. [26] found that HPV positive cases were found mainly among groups of T1 and T2 lesions with a maximum size of 50mm, and suggested that HPV could have an important role in growth pattern and metastatic potential of breast carcinomas with better prognosis. On the other hand, HPV was not found to have any prognostic value in breast carcinoma as proved by Francis [27].

In our study none of the triple negative cases were associated with HPV infection and the results were statistically not significant. However, there was a highly statistically significant positive correlation between HPV infection and Her2 neu positive cases. These results were unlike the study of done by Fernandes et al. [26] who reported that Her 2 positive cases were negative for HPV infection, while the association between HPV infection and triple negative cases reached a statistically significant value. While Piana et al. [28] recorded HPV positivity in 15% of cases of triple negative breast carcinoma in an Italian case control study. *W*e recommend more investigations on the link between molecular subtypes of breast carcinoma and susceptibility to HPV infection is recommended to be investigated.

Conclusion

Findings of the current study support the previously reported association between HPV and Breast cancer. Also suggests the role of HPV in tumor progression and spread.

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