Survey of Human Papilloma Virus 18 in Breast Epithelium of Iraqi women Using In situ Hybridization Technique

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ABSTRACT:

BACKGROUND:

There is an increasing evidence suggesting that high-risk human papilloma virus (HPV) was involved in cancers other than cervical cancer. A number of reports have identified HPV DNA in breast tissue and breast cancer specimens, suggesting that the virus could play a role in the pathogenesis of this tumor. Therefore,

OBJECTIVE:

was directed towards the use of In situ molecular methods to localize the virus in mammary gland tissue. In addition, this study investigated the prevalence of high-risk HPV18 infections in Iraqi women with and without ductal carcinoma of the breast.

PATIENTS AND METHODS:

Twenty night cases of ductal carcinoma patients and 44 controls obtained from adjacent area to benign breast lesions. Formalin fixed, paraffin embedded specimens were used by *In situ* hybridization technique for detection of HPV18 subtype. Data analysis was performed by SPSS 20 software using descriptive statistics and Chi-square tests.

RESULTS:

The HPV18 were identified in 65.5% and 20.5% of the ductal carcinoma and control breast tissue specimens respectively. Statistically, the difference between the normal and ductal carcinoma cases were highly significant (P=0.001

CONCLUSION:

Statistically, *In situ* hybridization revealed a significant increase of HPV18 in cases of ductal carcinoma (DC) (65.5%) when compared to their controls (20.5%) .

Most HPV were localized in the nuclei of integrative form.

HPV18 were detected in skin and mammary tissue of both ductal carcinoma and control cases. This may indicates that HPV18 has a role in ductal carcinoma pathogenisis.

KEY WORDS: human papillomavirus, breast cancer

INTRODUCTION:

Breast cancer, is the most common cancer and most common cause of death in middle aged women [(1)], and its incidence of was increased by more than 40% over 25 years [(2)]. The aetiology of breast cancer remains unknown. Many risk factors have been associated with the pathogenesis of this disease, including family history, hormones, cigarette smoking and alcohol consumption [(3-8)].

Hormones, cigarette smoking and family history have also been demonstrated to enhance infections with papillomaviruses, mainly the types of high-risk human papillomavirus (HPV) that involved in the aetiology of cervical carcinoma [(8)].

High-risk human papillomaviruses (HPVs) are important risk factors for numerous human cancers including cervical, colorectal and head and neck; roughly 96, 80 and 28% of these cancers are positive for high-risk HPVs, respectively [(9,10,11)]. Furthermore, Human papilloma viruses are accepted as being carcinogenic in human cervical and anogenital cancers [(12)]. Cervical cancer is caused by specific HPV infections [(13)]. The role of human papillomavirus (HPV) in breast carcinogenesis is controversial (14). It has been reported that high risk human papilloma viruses (HPVs) were present in more than 50% of human breast cancers [(15-23)]. Moreover, HPVs were

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detected in normal breast tissue in patients with in situ and invasive ductal carcinoma [24]. Furthermore, HPVs were elicited in milk of breast sample3 days post partum [polymerase chain reaction (PCR) study] [25]. On the contrary, Hedau *et al* (2011) [26] didn't elicit HPVs in cases of Breast cancer using polymerase chain reaction (PCR study).

MATERIALS AND METHODS:

Patients were selected from those who attending the operation room at "Baghdad teaching hospital-medical city complex" between January 2012 and November 2012. A total of 73 patients were involved in this study and they were divided into two groups (Table-1). Their age were ranged 16-68 years.

Table 1: Tissue categorization.

Group	Disease	Number of patients
I	Apparently Normal breast tissue (control)	44(60.3%)
II	Breast tissue with ductal carcinoma	29(39.7%)
Total		73(100%)

All samples were fixed in 10% buffered neutral formalin for about 20 hours, then embedded in paraffin (at 60 C˚) as blocks which sectioned into 5µm thick sections. These sections were mounted on charged slides. Then tissue sections were de-paraffinized. The DNA probe hybridization/detection system in situ kit were selected from Maxim bio (San Francisco, USA. Catalogue No. IH-60059(HPV-6011), IH-60001 (IHD-0050), and IH-60002 (IHD-0052). The procedure was used in accordance to the manufacturer instructions. Three control slides were employed: First one (positive control) is previously known to be strongly positive for the targeted genes (HPV). The second positive one was prepared by adding 20µl of housekeeping gene instead of the detecting probe (to specify the Kit is still working). While the third, the negative control was used by adding 20µl of PBS instead of the diluted probe. The three control tissue sections were necessary to keep fidelity in terms of specificity and sensitivity. Proper use of this hybridization/detection system in positive test tissue gave an intense blue signal at the specific site of hybridization probe.

In situ hybridization scoring system for HPV-RNA according to Alizi *et al* (2012) [27] was used in this study. This system calculates the percentages of epithelial cells with blue/black nuclear staining. Negative tissue sections were those of zero HPV-RNA expression cells. Quantification of different molecular markers in situ hybridization signal was evaluated under light microscopy and the counting of positive cells was performed at X100. Positive cells were counted in ten different fields of 100 cells for each sample and the average of positive cells of the ten fields was determined by assigning cases to one of the three following percentage score categories: 1-25%, 26-50, and >50 were considered as low, intermediate, and high positive respectively, Table (2).

Table 2: In situ hybridization scoring system for HPV-RNA (Alizi et al, 2012).

Evaluation	Negative	Low	Intermediat	High
Percentage of stained cells	zero	1-25%	26-50%	>50%

Chi-square test was used to detect the significances between variables of our study. All the statistical analysis was done by SPSS program (version-20). P-value was considered significant when < 0.05, & highly significant when < 0.01.

RESULTS:

28 cases out of 73 (38.4%) cases were detected to have positive HPV18 expression in their breast

tissue. The remaining 45 cases (61.6%) were found to express negative HPV18 (Table-3) and (Figure-1)

Table 3: HPV18-RNA expression in control and DC patients.

Cases	negative	positive HPV 18				Total	P-value
		low	intermediate	high	Total +ve		
Control	35(79.5%)	5(55.6%)	4(44.4%)	0(0%)	9(20.5%)	44	0.001
DC*	10(34.5%)	9(47.4%)	7(36.8%)	3(15.8%)	19(65.5%)	29	
Total	45(61.6%)	14(50%)	11(39.3%)	3(10.7%)	28(38.4%)	73(100%)	

*DC= ductal carcinoma of the breast.

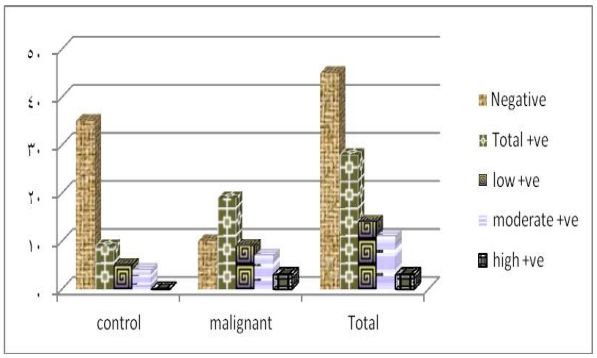
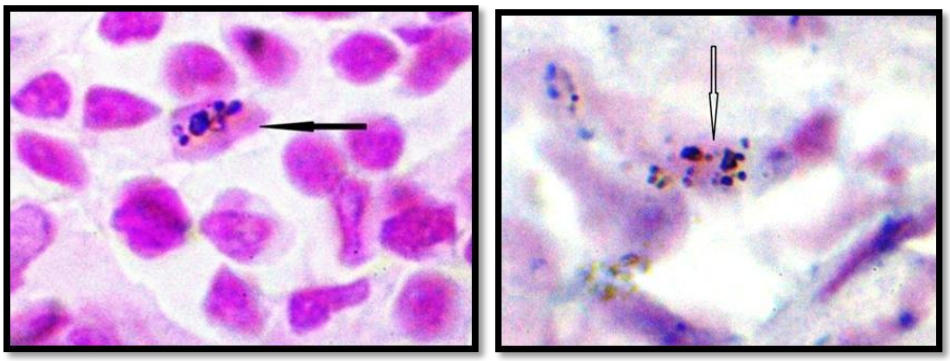


Figure 1: Histogram showing HPV18-RNA expression in each selected group.

The study detected that 79.5% (35/44) of control cases had negative HPV18-RNA expression. On other hand, 20.5% (9/44) of cases show positive HPV18-RNA expression. "Low" (Figure-2(A)) and "intermediate" (Figure-2(B)) HPV18-RNA expression nearly the same; representing 55.6% and 44.4% respectively. There was no high HPV18-RNA expression (Table-3) and (Figure-1).

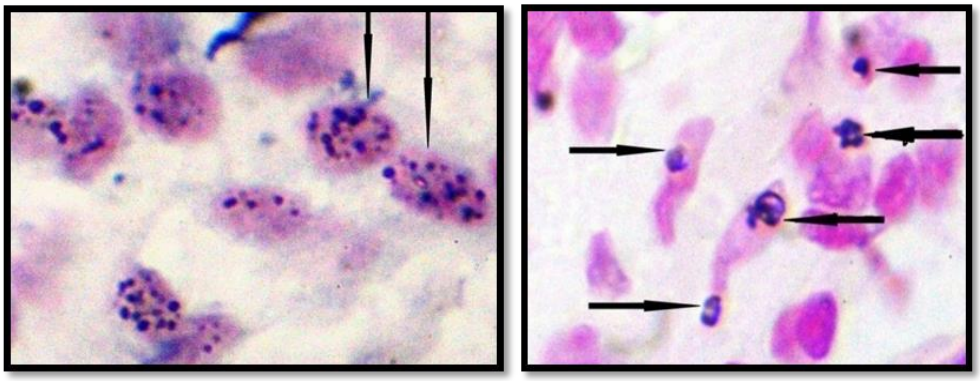
In 34.5% of DC cases there was no HPV18-RNA expression. The remaining (65.5%) of DC patients showed positive HPV18-RNA expression. "Low", intermediate (Figure-3(B)), and high (Figure-3(A)) HPV18-RNA expression were found to represents 47.4% (9/29), 36.8% (7/29), and 15.8% (3/29) respectively (Table-3) & (Figure-1).

Highly significant P-value (equals to 0.001) indicates the differences between apparently normal and malignant cases (Table-3).



(A) **(B)**

Figure 2: Two different Control breast tissue revealed: (A) "low" & (B) "intermediate" positive HPV18-RNA expression by ISH, stained by BCIP/NBT (bluish purple, arrows) and counter stained by NFR (X1000).



(A) **(B)**

Figure 3: Tow different patients with ductal carcinoma, the black arrows show: (A) "high" and (B) "intermediate" HPV18-RNA positive expression by ISH, stained by BCIP/NBT (bluish purple, black arrows) and counter stained by NFR (X1000).

DISCUSSION:

Human papillomaviruses belong to the papillomavirus family, *Papillomaviridae*. They were capable of infecting humans and they were the most prevalent cause of sexually transmitted viral infection. It is estimated that 80% of sexually active adults have been infected with at least one HPV type [28]. Beside HPV16, the second most important player of cervical cancers was the HPV18, which was found in 7-20% of cases [29].

Up to our best knowledge, this is the first study do scoring of HPV18 in the breast tissue all over the worlds.. In the present study, high percentages were found in the low score categories of HPV18. This may reflect a low reproduction (replication) rate of the virus [27] in mammary tissue.

There were few studies investigating the presence of HPV in an apparently normal breast tissue [18,19,23,30,31,32,33]. In the current study, HPV18 were reported in 20.5% (9/44) of the apparently normal breast tissue. This outcome almost coincides with that conducted by Heng *et al* (2009) [32]; Lawson *et al* (2009) (33); and Glenn *et al* (2012) (34), who recorded 18%, 22.2% and 20% prevalence of HPV18 in control breast tissue samples respectively. However, Taiwanian and Iranian researchers [30,35] elicited lower percentage (5% & 7.8%) respectively. On the contrary, many researchers failed to detect HPV18 in apparently normal breast samples [16,18,23].

In respect to ductal carcinoma patients, we observed HPV18 in 65.5% (19/29) of samples. This result parallels that recorded by Aceto *et al* (2010) (36); who elicited 60% HPV18 prevalence in breast cancer patients. Moreover, other researchers manifested HPV18 in 50% of breast cancer patients [12,34]. Higher percentage was arrayed by Lawson *et al* (2009)[33], who demonstrate HPV18 in 75% of patients with Ductal carcinoma of the breast. Lower

percentages were recorded previously [16,17,23,32,35,37]. Although it is well established that HPVs are the major causal agent for cervical cancer, involvement of these viruses in breast cancer is more controversial. This controversy may influenced by the technical limitations, different primer sets and/or detection probes, the epidemiology of HPV in different geographical area, different sexual behavioral patterns, differing incidences of anogenital HPV infection and possibly different population genetics could play a role in these differing results [35,39]. Moreover, the viral load of HPV in breast cancer appears to be extremely low. In a study of HPV in breast cancer of Japanese women, it was estimated that viral load of HPV in cervical cancer was 4,000 folds greater than that in breast cancer [37]. This renders the detection of HPV in the breast tissue more difficult and therefore may constitute to the absence of HPV in breast cancers reported by some investigators.

At present, about 130 types of HPV were identified by their sequence of the gene encoding the major capsid protein L1 isolated from HPV associated diseases. Moreover, HPVs can be also classified into high- and low-risk types depending upon their oncogenic potential. The high-risk HPV 16,18,31,33,35,45 associated with anogenital cancers and the precursor lesions (intraepithelial neoplasia), particularly of the cervix,. The most important players of cervical cancers are HPV16 and HPV18, found in 50–70% of cases [29]. This may indicate oncogenic role of HPV18 in breast cancers. The presence of high-risk HPV in both malignant and apparently normal breast samples implies their possible role in breast cancer carcinogenesis [14].

The oncogenic mechanisms by which HPV induces cervical cancer have been intensively studied [41]. High-risk HPV encodes a series of proteins, designated as early (E1–E7) or late (L1 and L2). Key of cellular transformation are the E6 and E7 oncoproteins, which work in concert to disrupt cell-cycle regulation, inhibit apoptosis and stimulate cell-cycle progression by binding/inhibiting the p53 and p110RB tumor suppressor genes, respectively [41]. Moreover, Xu *et al* (1995) [40] extensively studied the programmed cell death (apoptosis) in normal human mammary epithelial cells, in cells immortalized with human papillomavirus, and in mammary carcinoma cell lines. He propose that HPV16 E6 protein modulates degradation not only of p53 but also of p21 and perhaps other proteins involved in apoptosis. Also, Heng and coworkers showed that the oncogenic characteristics of HPV associated breast cancer are very similar to HPV-associated cervical cancer. Furthermore, two researches from different geographical areas demonstrate the same High-risk HPV types; 16 and 18, present in both breast and cervical cancers of the same patient [32]. These finding disclose a strong relationship of the High-risk HPV as a common factor in carcinogenesis of both cervical and breast cancers.

Statistical analysis of the current study disclosed (P-value = 0.001) high significance of HPV18 expression between the control and ductal carcinoma cases. This indicates a likely role of high-risk HPV18 in human breast cancer.

In addition to the oncogenicity of HPV18 in cervical cancers, the statistical outcome of the current study may indicate a role of HPV18 in DC pathogenesis. Further investigation with larger sample size and using different types of molecular techniques may needed to determine the exact role of HPV in pathogenesis of ductal carcinoma of the breast. Consequently, vaccination programs may be beneficiary.

REFERENCES:

1. Al-Shawi A A,. Descriptive Study of Breast Cancer in Missan (South of Iraq). Medical Journal of Babylon. 2012;9:419-26.
2. Howe HL, Wingo PA, Thun MJ, Ries LAG, Rosenberg HM, Feigal EG, Edwards BK: Annual report to the nation on the status of cancers (1973 through 1998), featuring cancers with recent increasing trends. *J Natl Cancer Inst.* 2001;93:824-42.
3. Longnecker MP, Bernstein L, Paganini-Hill A, Enger SM, Ross RK. Risk factors for *in situ* breast cancer. *Cancer Epidemiol Biomarkers Pre.*,1996;5:961-65.
4. Trentham-Dietz A, Newcomb PA, Storer BE, Remington PL: Risk factors for carcinoma *in situ* of the breast. *Cancer Epidemiol Biomarkers Prev.* 2000;9:697-3.
5. Band PR, Nhu DL, Fang R, Deschamps M: Carcinogenic and endocrine disrupting effects of cigarette smoke and risk of breast cancer. *Lancet.* 2002;360:1044-49.
6. Brinton L, Lacey J Jr, Devesa SS: Epidemiology of breast cancer. In *In Cancer of the Breast*. 5th edition. Edited by Donegan WL, Spratt JS. London: Saunders Elsevier Science. 2002:111-32.
7. Hamajima N, Hirose K, Tajima K, Rohan T, Calle EE, Heath CW Jr, Coates RJ, Liff JM, Talamini R, Chantarakul N, *et al.*: Alcohol, tobacco and breast cancer – collaborative

reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 96,057 women without the disease. *Br J Cancer* 2002;87:1234-45.

8. Hemminki K, Granström C, Czene K: Attributable risks for familial breast cancer by proband status and morphology: a nationwide epidemiologic study from Sweden. *Int J Cancer* 2002;100:214-19. zur Hausen H: Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* 2002; 2:342-50.

9. Damin DC, Caetano MB, Rosito MA, Schwartsmann G, Damin AS, Frazzon AP, Ruppenthal RD, Alexandre CO. Evidence for an association of human papillomavirus infection and colorectal cancer. Eur J Surg Oncol. 2007; 33: 569–74. [IVSL]

10. Sturgis EM & Cinciripini PM: Trends in head and neck cancer incidence in relation to smoking prevalence: an emerging epidemic of human papillomavirus-associated cancers?. Cancer. 2007;110: 1429–35.

11. De Sanjosé S, Diaz M, Castellsagué X, Clifford G, Bruni L, Muñoz N, and Bosch FX: Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis. Lancet Infect Dis. 2007;7:453–59.

12. Kan CY, Iacopetta BJ, Lawson JS, & Whitaker NJ: Identification of human papillomavirus DNA gene sequences in human breast cancer. Br J Cancer. 2005;93:946-48. [IVSL]

13. zur Hausen H. Papillomavirus infections: a major cause of human cancers. Biochem. Biophys. Acta.1996;1288: F55–F78.

14. Liang W, Wang J, Wang C, Lv Y, Gao H, Zhang K, Liu H, Feng J, Wang L and Ma R. Detection of high-risk human papillomaviruses in fresh breast cancer samples using the hybrid capture 2 assay. Journal of Medical Virology. 2013; 85:2087–92. [IVSL]

15. Yu Y, Morimoto T, Sasa M, Okazaki K, Harada Y, Fujiwara T, Irie Y, Takahashi E, Tanigami A, & Izumi K. Human papillomavirus type 33 DNA in breast cancer in Chinese. Breast Cancer. 2000;7: 33-36. [IVSL]

16. Liu Y, Klimberg VS, Andrews NR, Hicks CR, Peng H, Chiriva-Internati M, Henry-Tillman R, & Hermonat PL. Human papillomavirus DNA is present in a subset of unselected breast cancers. J Hum Virol. 2001;4:329–34.

17. Damin APS, Karam R, Zettler CG, Caleffi M, & Alexandre C. Evidence for an association of human papillomavirus and breast carcinomas. Breast Cancer Res Treatment. 2004;84:131–37.[IVSL]

18. De Villiers E.M., Sandstrom R.E., Hausen H.Z., and Buck C.E. Presence of papillomavirus sequences in condylomatous lesions of the mamillae and in invasive carcinoma of the breast. Breast cancer research. 2005;7:R1-R11.

19. Lawson JS, Guenzburg W, & Whitaker NJ. Viruses and breast cancer. Future Microbiol. 2006a;1:33–51.

20. Lawson JS, Kan CY, Iacopetta BJ, & Whitaker NJ. Are some breast cancers sexually transmitted? Br J Cancer. 2006b; 95: 1708.

21. Yasmeen A, Bismar TA, Kandouz M, Foulkes W, Desprez P-Y, Al Moustafa A-E. E6/E7 of HPV type 16 promotes cell invasion and metastasis of human breast cancer cells. Cell Cycle. 2007a;6: 2033–42.

22. Yasmeen A, Ricciardi R, Kassab A, Bismar TA, Al Moustafa A-E. High-risk HPVs in human breast cancer and normal mammary tissues. The Breast J.2007b;16:445.

23. De León DC, Montiel DP, Nemcova J, Mykyskova I, Turcios E, Villavicencio V, Cetina L, Coronel A, & Hes O. Human papillomavirus (HPV) in breast tumors: prevalence in a group of Mexican patients. BMC Cancer.2009; 9:26-32. [IVSL]

24. Lawson J S, Heng B , Glenn W K , Ye1 Y , Tran B, Delprado W, Lutze-Mann L, and Whitaker N J. Human papilloma virus is associated with breast cancer. BJC. 2009a;101:1345–50.

25. Marja S , Marjut R, Seija G, & Stina Sl. Human Papillomavirus DNA Detected in Breast Milk. Pediatric Infectious Disease Journal. 2008; 27:557-58.

26. Hedau S, Kumar U, Hussain S, Shukla S, Pande S, Jain N, Tyagi A, Deshpande T, Bhat D, Mir MM, Chakraborty S, Singh YM, Kumar R, Somasundaram K, Bharti AC, and Das BC. Breast cancer and human papillomavirus infection: no evidence of HPV etiology of breast cancer in Indian women. BMC Cancer. 2011;20: 11-27.[IVSL]

27. Alizi S, Mukhlis F, & Abdul-Majeed B, 2012. Detection of Human papillomavirus in surface epithelial ovarian carcinoma using in situ hybridization technique. Fac Med Baghdad. 2012;54:57-62.

28. Baseman J.G. and Koustsky L.A.. The epidemiology of human papillomavirus infections. J. Clin. Virol. 2005;32:16–24.

29. Stanley M. Pathology and epidemiology of HPV infection in females. Gynecol Oncol.2010;Suppl 2: 5–10. [IVSL]

30. 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; 75:276-81. [IVSL]

31. He Q, Zhang SQ, Chu YL, Jia XL, & Wang XL. The correlations between HPV16 infection and expressions of c-erbB-2 and bcl-2 in breast carcinoma. Mol Biol Rep. 2009;36:807-12.

32. Heng B, Glenn W K, Ye Y, Tran B, Delprado W, Lutze-Mann L, Whitaker N J and Lawson J S. Human papilloma virus is associated with breast cancer, British Journal of Cancer. 2009; 101: 1345–50. [IVSL]

33. Lawson J S , Glenn W K , Heng, Ye Y, Tran B, Lutze-Mann L and Whitaker N J. Koilocytes indicate a role for human papilloma virus in breast cancer. British Journal of Cancer. 2009a;101:1351–56. [IVSL]

34. Glenn WK, Heng B, Delprado W, Iacopetta B, Whitaker NJ, & Lawson JS. Epstein-Barr virus, human papillomavirus and mouse mammary tumour virus as multiple viruses in breast cancer. PLoS One. 2012; 7: e48788. [IVSL]

35. Manzouri L, Salihi R, Shariatpanahi S, & Rezaie P. Prevalence of human papillomavirus among women with breast cancer since 2005-2009 in Isfahan. Adv Biomed Res. 2014;3:75.

36. Aceto GM, Solano AR, Neuman MI, Veschi S, Morgano A, Malatesta S, Chacon RD, Pupareli C, Lombardi M, Pattista P, Marchetti A, Mariani-Costantini R, & podesta EJ. High-risk human papilloma virus infection, tumor pathophenotypes, and BRCA1/2 and TP53 status in juvenile breast cancer. Breast Cancer Res Treat. 2010;122:671-83. [IVSL]

37. Khan NA, Castillo A, Koriyama C, Kijima Y, Umekita Y, Ohi Y, Higashi M, Sagara Y, Yoshinaka H, Tsuji T, Natsugoe S, Douchi T, Eizuru Y, & Akiba S. Human papillomavirus detected in female breast carcinomas in Japan. Br J Cancer.2008;99: 408-14.[IVSL]

38. Suarez AL, Lorenzetti MA, Gonzalez Lucano R, Cohen M, Gass H, Vazquez PM, Gonzalez P, . Preciado MV, & Chabay P. Presence of human papilloma virus in a series of breast carcinoma from Argentina. PLoS One. 2013;8: e61613.

39. Mallia R, Mangion JP, CamenzuliC, Cassar A, Cacciottolo P, Cauchi J, Borg J, Ali S, and Schembri-Wismayer P. HPV positivity varies with technique and primer set, in formalin-fixed paraffin-embedded benign and malignant breast tissue from Malta. American Journal of Molecular Biology. 2011;1:183-88. [IVSL].

40. Xu C, Meikrantz W, Schlegel R, and Sager R. The Human Papilloma Virus 16E6 Gene Sensitizes Human Mammary Epithelial Cells to Apoptosis Induced by DNA Damage. National Academy of Sciences. 1995;92:7829-33. [IVSL]

41. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. Nature Publishing, Group NATURE REVIEWS, CANCER. 2002;2:343-44.