Detection of Human Papilloma Virus in Iraqi Patients with Breast Cancer

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ABSTRACT:

BACKGROUND:

It is well realized that breast cancer is the most terrifying cancer for females, the commonest malignancy and the second leading cause of cancer death in women. Recent studies suggested that the association of viral infection with breast cancer pathogenesis such as Epstein Barr virus and mouse mammary tumor virus. In addition, human papilloma virus DNA sequences have been isolated. There is relationship between human papilloma virus and other neoplasms (anogenital, skin and 99.7% of cervicouterine carcinomas).

OBJECTIVE:

This study has been undertaken for detecting high risk group HPV genotypes in breast carcinoma in different provinces in Iraq depending on molecular technique.

PATIENTS AND METHODS:

A total of 59 patients include both sexes (male and female) diagnosed histopathologically as having breast cancer were included and studied during the period from January to August, 2010 in different regions in Iraq. In addition to 20 cases of benign breast lesion used as a control group. Theses samples were represented by formalin-fixed paraffin embedded blocks and fresh frozen tissue. A multiplex PCR analysis were done for the DNA extracted from these samples.

RESULT:

The mean age in this study was 45.5 years. Ninety three percentage were female and the remaining were male. Fourty percentage of tumor present in the right side, 59.3% removed by lumpectomy. Eighty six percentage were found to have infiltrating ductal carcinoma followed by infiltrating lobular carcinoma (13.8%). Twelve HPV genotypes were used in this study which of high risk group including (16,13,33,35,18,45,39,59,52,56,58 and 66)**.** HPV type 39,59 and 52 were demonstrated among those patients and the result show about 16.9% of our patients (10 out of 59) had positive HPV demonstrated by PCR in their breast tissue and 83.1% (49 out of 59) were negative for HPV. All the benign breast cases were negative for HPV except one case of fibrocystic disease which was positive for HPV type 39 which was not significant (*p* value >0.05).

CONCLUSION:

The study concluded that high risk group Human Papiloma virus genotypes 39, 59 and 52 genotype were present in breast cancer tissue in Iraqi patients.

***KEY WORDS*:** human papiloma virus, breast cancer, PCR.

INTRODUCTION:

Breast cancer is the most common non –skin malignancy in women. The incidence increase with age, raising concern that was an un identified environmental cause.[1] Breast cancer is relatively slow growing tumors and the most frequently diagnosed malignancy of women in many populations. Research into its etiology has focused primarily on reproductive and other factors affecting circulating sex hormones, and on genetic susceptibility. Hormone, as identified risk factors thought to explain only about half of all breast cancer incidences. Researches are motivated to consider other routes of disease pathogenesis[2].

As in all cancers, the cause of breast cancer remains unknown. Cancer can be defined as a cellular disorder characterized by progressive accumulation of a mass of cells, as a result of

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excessive production of cells not compensated by appropriate cell loss. Correlation of DNA identification of HPV and breast cancer ranges in variability from 0-86% of cases [3]. The mechanism by which the virus reaches the breast has not been clearly identified [4].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 cancer [5]. The immortalization of normal breast epithelial cells by the HPV 16 and 18 has been used to study the functions of the viral early genes E6 and E7 in different cellular pathways [6, 7].

PATIENTS AND METHODS:

PATIENTS:

A total of 59 patients include both sex (male and female) diagnosed histopathologically as having breast cancer were included and studied during the period from January to August 2010. They included 34 patients were diagnosed retrospectively and 15 patients were diagnosed prospectively. These were represented by formalin-fixed, paraffin embedded tissue. In addition to that, 10 patients were diagnosed at al-Ramadi teaching hospital represented by fresh tissue preserved in normal saline and kept in -20 ^{o}C. Collected specimens were obtained from different cities of Iraq as follow: Ten patients (16.9%) were diagnosed at Medical City Teaching Hospital (Baghdad), ten patients (16.9%) diagnosed at Arbil hospital, twelve patients (20.4%) diagnosed at private laboratory in Al-Basraha, fourteen patients (27.3%) diagnosed at private laboratory in Al-Ramadi, and thirteen patients (22.1%) diagnosed at private laboratory in Al-Faluja.

Control group: another group of 20 patients diagnosed as having benign breast lesions (fibrocystic disease, fibro-adenoma, ductectesia) were also included in this study as a control group. These specimens was obtained either by excisional biopsy (lumpectomy) or mastectomy. This work was done in molecular laboratory in college of medicine /Al-anbar university.

The blocks are sectioned at 5 micron thickness (10-20X) and preserved in Eppendroff tube for the molecular analysis. Reassessment was performed for tumor typing and grading according to WHO classification [8]. The stage of the tumor was carried out according to American Joint Committee on Cancer Staging, 2002 [9] to assess the extent of the tumor**.**

DNA Extraction from formalin fixed paraffin embedded tissue:-

DNA was extracted from formalin fixed paraffin embedded tissue as follows:-

Day 1 :

1. 1 ml of xylol added (pre incubated at 37 ^{o}C for 1hr) incubate at RT for 30min.
2. Vortexing for 1 minute
3. Spin down for 5min ,13000rpm and supernatant was discarded.
4. repeat the step 1,2,&3.
5. 1ml 100% ethanol added, incubated for 30min
6. Vortexing for 1 minute.
7. Spin down for 5min ,13000rpm and supernatant was discarded.
8. 1ml 75% ethanol added, incubated for 30min
9. Vortexing for 1 minute.
10. Spin down for 5min ,13000rpm and supernatant was discarded.
11. 10µl of PK added (stock solution 20 mg/ml) , incubate over night at 55˚C in shaker water bath.

DAY 2:

١٢. 10µl of PK added , incubated over night at 55˚C in shaker water bath.

DAY 3:

13. 25µl of PK added , incubated over night at 55˚C in shaker water bath.

DAY 4:

14. 25µl of PK added , incubated over night at 55˚C in shaker water bath.

DAY 5:

15. 500 µl of PCI added ,incubated for 5min at RT.
16. Spin down for 5min at 13000rpm. Supernatant was taken in new tube
17. Add 500 µl of PCI ,shaked gently and incubated for 10 min at RT.
18. Spin down for 5min at 13000rpm, the supernatant were collected in new tube
19. 300 µl 7.5M ammonium acetate, 1ml cold 100% ethanol and 5 µl glycogen (if present) (stock 20mg) were added, shaked gently and incubated at -20 for 2hr or overnight
20. Spin down for 30min at 13000rpm and supernatant was discarded.
21. Air dry pellet for 10min at RT
22. 25 µl deionized distilled water added and incubated at -20 until the samples were used.

DNA Extraction from Fresh frozen tissue:-

DNA was extracted from fresh frozen tissue as follows:

1. **Breast tissue thawing**: the pre-cut samples were taken out of the (-20) freezer, thawed at RT.

2.**Tissue processing**: one gram of frozen tissue was cut to a very fine piece by using surgical blade & a new blade used with each sample.

3.Tissue washing: 10 ml of phosphate buffer solution (PBS) was added to each tissue piece in a tube, each tube was inverted to make sure that all the tissue was in the solution & not stick to the tube walls. Spine down at 1400 rpm for 5 min.

4.**Tissue digestion** : the supernatant removed carefully, and 0.5 ml PK added. The pellet re suspended by vortexing, and the tubes were placed in incubator at 55˚C with shaking over night. The day next we made sure that all the tissue has been digested, if not, more PK were added & allowed for digesting for a few more hours.

Each tube was split into two 1.5ml eppindrof tubes.

5.**DNA purification** : equal volume (500 µl) of PCI was added into each tube and centrifuged for two min. at 1400 rpm. The aqua's layer removed, placed into a new tube.

6.**DNA concentration** : the aqueous phase was aliquot into plain tube, 5 ml of absolute ethanol and 1/10 of the volume (7.5 M) ammonium acetate were added and freeze over night at (-20˚C). The DNA pellet was transferred into 1.5ml eppendrof tube after centrifugation at 1400 rpm for 15 min.

7.**DNA washing**: 1 ml of 100% ethanol was added, mixed and centrifuged at 1400 rpm for 15 min. the supernatant was dumped & air dried pellet.

8.**DNA suspending**: the DNA was dissolved in 100 µl nuclease free water (injection water) & left at room temperature over night. Then DNA concentration measured and the DNA was stored at -20˚C. [10]

DNA quantitation:-

DNA concentration was measured according to the equation:

DNA concentration (µg/µl) = [OD260 X 100 (dilution factor) X50 µg/ml] /1000.

Theoretically, OD260 of one corresponds to approximately (50µg/ml) for double strand DNA. The ratio between the reading at 260nm and 280nm (OD260/OD280) provides an estimation of the purity of nucleic acid. [11] Pure DNA will give an A260/A280 of 1.8 or higher. Values of A260/A280 of less than 1.8 indicate contamination of the DNA by protein. For good PCR results, DNA is required with an A260/A280 quotient of 1.6 or greater. [12,13]

Polymerase chain reaction:

Principle of the assay:

In this technique, genomic DNA is first heated and denaturated to form single strands. In the annealing phase, the DNA is cooled, allowing hybridization with primer sequences that flank the region of interest. Then the reaction is heated to an intermediate primer extension temperature, in which DNA polymerase add free bases in the 3'direction along each single strand, starting at the primer. Blunt- ended DNA fragments are formed, and these provide a template for the next cycle of heating and cooling. Repeated cycling produces a large number of DNA fragments bounded on each end by the primer sequence [14].

Procedure:-

HPV high risk typing kit (saccace, Italy) was used for amplifying the target genes as follow: E6, E2, E1 and L1 genes as follow:-

2 µl of template DNA and 2 µl of primers were added to PCR pre-mix tube. Afterthat, distilled water was added to PCR tubes to total volume of 20 µl. Vortexing and briefly spin down (to dissolve the lyophilized blue pellet),then mineral oil was added. The PCR tray which contained the reaction mixture was transferred into the thermocycler. The PCR was started as in the following steps:

1. Pre-heated at 94Cº for 2 minutes.
2. Fourty cycles for HPV. Each cycle consist of the following steps:-

a. Denaturation at 94 ºC for 30 second.
b. Annealing at 60 ºC for 45 seconds.
c. Extension at 72 ºC for 1 minute.

3. An incubation or final extension at 72 ºC for 5 minutes[15].

The PCR products were detected by agarose gel electrophoresis as mentioned in the following section and stained by ethidium bromide. The target bands were detected by ultra violet light with use of UV transilluminator.

Preparation:-

1. 10 ml of TBE10X (0.445M Tris-Borate, 0.0125 M EDTA) added to 90ml sterile distilled water
2. 2gm agarose gel dissolved in 100ml TBE 1X and prepared by boiling until the solution become clear. The solution allowed to cool to below 60 Cº. Then poured into toped plate to make gel.
3. After polymerization (about 30 minutes at room temperature), the comb and tape were removed. The gel was placed into the gel chamber that was filled with 1X TBE buffer.

The gel pocket should be completely covered with buffer.

4. Entire PCR mixture (10µl) was pipette into the gel pockets.
5. Electrophoresis was then carried out for about 1 hour with the following conditions (5 volt/cm, 100w and 60mA).
6. When the electrophoresis was completed; the gel was placed in a tank of staining containing Ethidium bromide (0.2gm of ethidium bromide was dissolved in 20ml of distilled water and stored at 2-8 C° and protected from light). Then 0.5µg/ml concentration was prepared according to equation C1V1=C2V2
7. After that the gel was placed on a UV transilluminator (suitable face protection against UV radiation should be worn)
8. A digital picture was made for evaluation and documentation of the results [11, 13].

Statistical analysis:

Statistical analysis was carried out using Pearson's Chi-square analysis and cross tabulation to assess significant difference using the computer program Microsoft excel 2003 and SPSS version 10. In this statistical analysis, a *p* value < 0.05 was considered to be significant [16].

RESULTS:

Twelve HPV genotypes were used in this study which are of high risk group including (16,13,33,35,18,45,39,59,52,56,58 and 66**).** HPV types 39, 59 and 52 were demonstrated among our patient and the result show that 16.9% of our patients (10 out of 59) had positive for HPV demonstrated by PCR in their breast tissue as shown in figure (2,3) and 83.1% (49 out of 59) were negative for HPV as shown in figure (1). All the benign breast cases were negative for HPV except one case of fibrocystic disease which was positive HPV type 39 as shown in (table 1) which was statistically not significant (*p* value >0.05).

Table 1: HPV genome among study groups

Study group		Positive	Negative	Total
Patient group	no.	10	49	59
(%)		(16.9)	(83.1)	(100)
Control group	no.	1	19	20
(%)		(5)	(95)	(100)
Total	no.	11	68	79
(%)		(13.9)	(86.1)	(100)
P value =0.182				

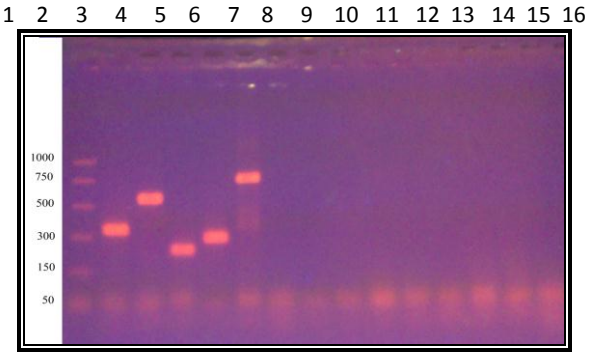

Figure (4-6): 3% agarose gel electrophoresis of PCR- amplicons of HPV genotypes (16,31,33,35) from formalin fixed paraffin embedded breast tissues , run with 5 volt/cm. All the cases were negative for these genotypes.

Lane 1 represents PCR markers.
Lane 2 represents positive control of HPV 16
Lane 3 represents positive control of HPV 31
Lane 4 represents positive control of HPV 33
Lane 5 represents positive control of HPV 35
Lane 6 represents internal control
Lane 7 represents negative control
From lane 8-16 represent the samples which were all negative for these 4 genotypes of HPV.

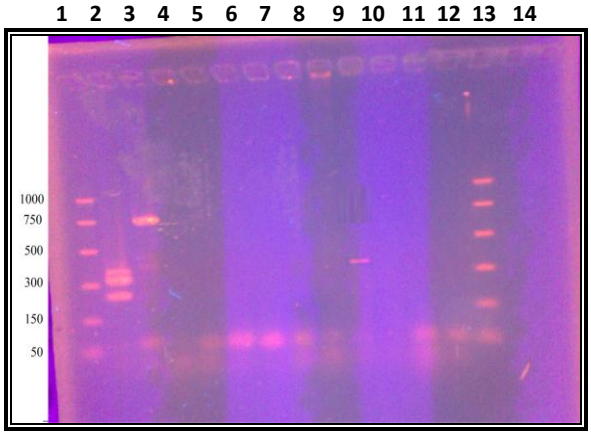

Figure (4-7): 3% agarose gel electrophoresis of PCR- amplicons of HPV genotypes (52 , 56, 58, 66) from formalin fixed paraffin embedded breast tissues , run with 5 volt/cm..

Lane 1 and 14 represent PCR marker
Lane 2 represents positive controls for HPV genotype (52,56,58,66)
Lane 3 represents internal control.
Lane 4 represents negative control.
Lanes 5-13 represent DNA extracted from formalin fixed paraffin embedded tissue of breast cancer.
Lane 10 demonstrate a band at 360 bp which is compatible to molecular weight of HPV type 52.

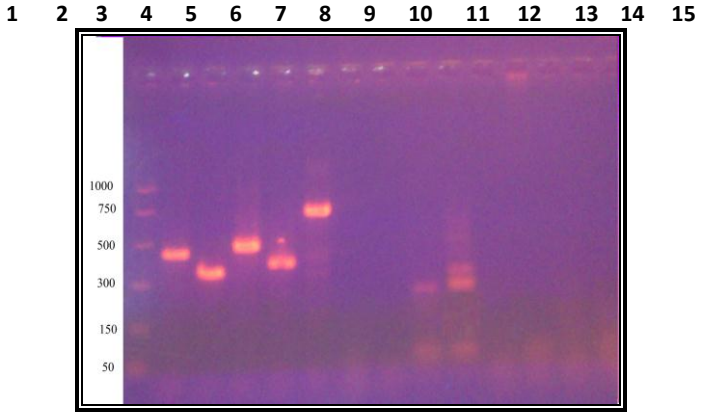

Figure (4-8): 3% agarose gel electrophoresis of PCR-amplicons of HPV genotypes (18,39,45,59) from formalin fixed paraffin embedded breast tissues , run with 5 volt/cm.

Lane 1 represents PCR marker.
Lanes 2 represents positive control of HPV type 18
Lanes 3 represents positive control of HPV type 39
Lanes 4 represents positive control of HPV type 45
Lanes 5 represents positive control of HPV type 59
Lane 6 represents internal control.
Lane 7 represents negative control.

Lanes 8-15 represent DNA extracted from formalin fixed paraffin embedded tissues of breast cancer.

Lane 9 shows band at 340 bp which was compatible to molecular weight of HPV type 39.

Lane 10 shows two bands at 340 bp and 395 bp which were compatible to molecular weight of HPV types 39 and 59 respectively.

DISCUSSION:

In Iraq, breast cancer is considered the most common type of malignancy among women accounting for about one third of the registered female cancers. [17] This could be attributed to hereditary, environmental and life style factors. Most of Iraqi patients are diagnosed in younger age groups with late stage at presentation and a prevalence of more aggressive tumor behavioral forms. [18]

In this study, HPV type 39,59 and 52 were demonstrated and the result show that 16.9% of our patients (10 out of 59) had positive HPV demonstrated by PCR in their breast tissue and 83.1% (49 out of 59) were negative for HPV. All the benign breast cases were negative for HPV except one case of fibrocystic disease which was positive HPV type 39 which was statistically not significant. Francisco et al., [19]in Chile demonstrated that HPV-16 was detected in 4/46 (8.7%) of breast cancer specimens.

It is well known that the transforming gene products of HPV have also been shown to immortalize breast epithelial cells *in vitro*. The observation of complete absence of HPV DNA sequences in breast cancer refute the possibility of any role for oncogenic genital HPV types 16 and 18 in the pathogenesis of breast cancer [20].

In India , All biopsies and blood samples of breast cancer patients tested by PCR methods did not show positivity for HPV DNA sequences in conventional PCRs for HPV16 E6/E7 primers [21] Others demonstrated Papillomavirus DNA was present in 25 of 29 samples of breast carcinoma. The most prevalent type was HPV 11, followed by HPV 6. Other types detected were HPV 16, 23, 27 and 57 [22].

Though other studies from around the world have also found a relationship between HPV and breast cancer, some have been criticized for the methods used and the fact that they have often led to mixed results (with some studies reporting the incidence of HPV-positive cells to be as low as 4% and negative as up to 86%) [23].

In Chinese women, DNA derived from HPV33 was detected in 14 cases (43.8%) out of 59. No HPV16 or HPV18 DNA was detected in any of the cases in this study. This is the first report demonstrating a correlation between HPV33 infection and breast cancer [23].

In the group of carcinomas 36 (70.5%) were negative and 15 (29.4%) were positive to HPV-DNA, 10(66.6%) were positive for HPV 16, 3(20%) for HPV 18, two cases (13.4%) were positive for both. In the group of benign conditions all were negative to HPV-DNA. [25]

Other study show that HPV 18 gene sequences are present in DNA extracted from breast tumors in Australian women. Overall, 24 (48%) of the 50 samples were HPV positive. Human papilloma viruses may have a role in human breast cancer. They speculate that HPVs may be transmitted by hand from the female perineum to the breast [26].

A study has shown that no HPV-related DNA sequences were identified. It therefore seems unlikely that a significant percentage of human breast carcinomas is etiologically related to infection with one of these HPV types [27].

CONCLUSION :

1. The study concluded that high risk group Human Papiloma virus genotypes 39, 59 and 52 genotype were present in breast cancer tissue in Iraqi patients where as other studies demonstrating other genotypes like HPV type 16,18.
2. Half of the cases of HPV positive were infiltrative ductal carcinoma and other half were infiltrative lobular carcinoma.

REFERENCES:

1. Robbin and Cotran: Pathologic Basis of Disease 8th edition, 2010:1129-54.
2. Glaser SL, Joe L, Hsu and Margaret L. Epstien – barr virus and breast cancer: state of the evidence for viral carcinogenesis cancer epidemiology biomarkers and prevention 2004;13:688-97.
3. De Cremoux P, Thioux M, Lebigot I, Sigal – Zafrani B, Salmon R, Sastre-Garau X, For the Institute Curie Breast Group: No evidence of human papillomavirus DNA sequence in invasive breast carcinoma. Breast Cancer Res Treat 2007.
4. Damin A, Karan R, Zettler CG, Caleffi M, Alexandre C: Evidence for an association of human papillomavirus and breast carcinomas Breast Cancer Res Treat 2004;84:131-37.

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

6. Liu Y ,Chen JJ, Gao Q, Dalal S, Hong Y, Mansur CP, Band V, Androphy EJ: Multiple functions of human papillomavirus type 16 E6 contribute to the immortalization of mammary epithelial cells. J Virol 1999;73:7297-7.

7. Helt AM, Funk JO, Galloway DA: Inactivation of both retinoblastoma tumor suppressor and p21 by human papillomavirus type 16 E7 oncoprotien is necessary to inhibit cell cycle arrest in human epithelial cells. J Virol 2002;76:10559-68.

8. World Health Organization,Histological typing of breast tumors, 2nd edition. Am J Pathol. 1982;78-86.

9. AJCC Cancer Staging Manual, 6th ed. New York, Springer, 2002.

10. Mullis and Malins K. DNA extraction from frozen tissue sections. The Mind Field 2003; 06:7977-79.

11. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harber Laboratory. New York, 2nd ed 1989.

12. Al- Shummari AHN. Brain Astrocytoma: human leukocyte antigen (HLA) genotyping and tumor suppressor gene (p53) genetic alterations detected by polymerase chain reaction(PCR/SSP-SSCP). Ph.D. thesis. College of Medicine, University of Al-Nahrain, 2004.

13. Mushtak TS Al-ouqaili. Molecular, bacteriological and immunological aspects in the diagnosis of human brucellosis. Ph.D. thesis. College of Medicine, University of Baghdad.2006.

14. Bricker BJ. PCR as a diagnostic tool for brucellosis. Vet Microbiol 2002;90:435-46.

15. Morata PM, Queipo-Ortuno MI, Colmenero JD. Strategy for optimizing DNA amplification in a peripheral blood PCR assay used for diagnosis of human brucellosis. J Clin Microobiol 1998;36:2243-46.

16. Chap TLE. Introductory biostatistics. By John Wiley & Sons, Inc./New Jersey,2003.

17. Results of the latest Iraqi Cancer Registry, Ministry of Health. Baghdad -Iraq 2004.

18. Al-Alwan N.A.S.: Clinicocytopathological evaluation of nuclear DNA ploidy and hormone receptor contents in breast tumors, a PhD thesis submitted to the college of medicine and the committee of post graduate studies of the University of Baghdad, 1998.
Francisco Aguayo **,** Noureen Khan**,** Chihaya Koriyama et al. Human papillomavirus and Epstein-Barr virus infections in breast cancer from chile. *Infectious Agents and Cancer* 2011, 6**:**7doi:10.1186/1750-9378-6-7.

19. Gopalkrishna V, Singh UR, Sodhani P, Sharma JK, Hedau ST, Mandal AK, Das BC.Absence of human papillomavirus DNA in breast cancer as revealed by polymerase chain reaction. Breast Cancer Res Treat. 1996;39:197-202.

20. Suresh Hedau, Umesh Kumar, Showket Hussain, Shirish Shukla et al. Breast cancer and human papillomavirus infection: No evidence of HPV etiology of breast cancer in Indian women.2011 January 20. doi: 10.1186/1471-2407-11-27.

21. Ethel-Michele de Villiers **,** Robert E Sandstrom **,** Harald zur Hausen and Charles E Buck . Presence of papillomavirus sequences in condylomatous lesions of the mamillae and in invasive carcinoma of the breast. *Breast Cancer Res* 2005, **7:**R1-R11doi:10.1186/bcr940.

22. Alice G. Walton et al. Vaccinating for HPV May Also Prevent Breast Cancer, Study Finds.2009.

23. Yingyan Yu, Tadaoki Morimoto, Mitsunori Sasa, Kuniyasu Okazaki, Yosuke Harada, Tsutomu Fujiwara, Yasuo Irie, Ei-ichi Takahashi, Akira Tanigami and Keisuke Izumi. Human papillomavirus type 33 dna in breast cancer in Chinese. Breast Cancer ,2000;7 : 10.1007/BF02967185.

24. David Bracey, "UC Scientist Wins 'American Nobel' Research Award. 2004 "University of Cincinnati press release.

25. C-Y Kan, B J Iacopetta, J S Lawson and N J Whitaker. Identification of human papillomavirus DNA gene sequences in human breast cancer. *British Journal of Cancer* 2005;93:946–48.

26. Bratthauer GL, Tavassoli FA, O'Leary TJ. Etiology of breast carcinoma: no apparent role for papillomavirus types 6/11/16/18. Department of Cellular Pathology, Armed Forces Institute of Pathology, Washington, DC.1992.