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Genotyping of Human Papilloma Virus *(HPV)* Isolated from Breast Cancer Patients in Radiation and Isotopes Center Khartoum *(RICK)*–Sudan

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Abstract

Background: *Breast cancer is the most common among female comprises about (18%) of all female cancers, with (1.7) million new cases in the world each year. Recently some studies reported that approximately (18%) of cancer cases can be linked to infectious agents including viruses particularly Human Papilloma Virus (HPV).*
Objectives: *The objectives of this study were to investigate of which genotype of HPV is implicated as well as the risk factors associated with HPV in breast cancer by using PCR as diagnostic tools for HPV in breast tissues.*
Materials and methods: *One hundred forty three females diagnosed with histopathological examinations to have breast cancer (n=100 patients) or breast inflammatory conditions (n=43patients) were employed in this study. Tissue sample of (10) µm was taken from the pathological tissues of each patient. The samples were subjected to a PCR protocol to determine whether the patient is infected with HPV or not. HPV infected samples were further subjected to anther PCR protocol to identify the genotypes of HPV. The risk factors associated with breast cancer were taken from patient's records. The data were subjected to analytical and descriptive statistical analysis.*
Results & Discussion: *Out of the cases screened for breast pathology, 41(29%) patients were found to have, invasive ductal carcinoma, 21(15%) invasive lobular carcinoma, 12(8%) invasive micropapillary carcinoma, 26(18%) medullary carcinoma and 43(30%) have inflammatory breast conditions.*
Genotyping of HPV infected breast cancer specimen was performed. Out of the (143) specimens (breast cancer and breast inflammatory conditions) the following genotypes were determined and mentioned in a descending order. 22(33%) of the HPVs were of the genotype 16, 21(31%) genotype 18=14(21%) genotype 33=14(21%) and 10(15%) genotype (31).

Conclusion: *In conclusion, the findings of this study provide strong association between HPV genotypes and BC in Sudan.*

Keywords: *HPV, Breast cancer, RICK, Sudan, PCR, Cell cycle.*

Introduction and Literature Review

Human Papilloma Virus

HPV is a member of the *papillomaviridae* family and have double stranded circular *DNA genome* icosahedral nucleocapsid. [2] These viruses are small in size with *8kbp-long DNA genome*, no enveloped virus. [2] *HPV genome* contains early *(E)* and the late genes *(L)* which codes for early proteins *(E1-E7)* late proteins *(L1 and L2*) and a non-coding *long control region (LCR*). [2] There are more than one hundred different *HPV types* that have been discovered and these were divided into high risk and low risk types. [2] *HPV* (16, 18, 31, 33, 35 45) was classified as high risk *HPV types* associated with many of the cancer while *HPV (6*, 11) are low risks non oncogenic *HPV types* [2, 3]

Classification of *Human Papilloma Virus*

Table (1.1) Some high risk, low risk and potentially risks *HPVs*. [2, 3]

Classification	HPV types
High-risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59.
Low-risk	6, 11, 40, 42, 43, 44, 54, 61, 70.
Potentially high –risk	26, 53.

HPV genome normally was founded in the cytoplasm of infected tissues however the *DNA* of *HPV types* that associated with cancer is integrated into the host genome. [6] *HPV was* caused disruption and loss of some of the viral genes *(L1 and L2 genes)* and also increases the

expression of the early genes. [7] *Onco-proteins (E5)* interacts with *MHC class I* and prevents its transport to the cell surface therefore infected cells escapes from the immune system consequently allowing the virus to establish persistent infections and thus progressing to cancer. [8] *E6 targets p53* for degradation and therefore prevents apoptosis of abnormal cells, whereas *E7* inactivates *Rb* function, which results in abnormal cell proliferation and disturbs the normal cell cycle regulation. [8, 9] *P53* and *Rb* are tumor suppressor genes which stop tumors from developing.

Function of Different Genes of *HPV*

Table (1.2) Function of different genes of *HPV* [133,134]

Gene/Region	Function
E1/E2	Code for proteins which control the function of *E6 and E7 genes*
E4	Function largely unknown but may control virus release from cell
E5	Codes for a hydrophobic protein which enhances immortalization of the cell
E6	Codes for proteins which inhibit negative regulators of the cell cycle .E6 products inhibit p53 which is a transcription factor for apoptosis
E7	Codes for products which bind to the retinoblastoma tumor suppressor proteins there by permitting the cell to progress through the cell cycle in the absence of normal mitogenic signals
L1/L2	Code for structural proteins and formation of complete virus particles
LCR	Necessary for normal virus replication and control of gene expression

Association of *HPV* with the Breast Cancer

The association of *HPV* with cervical cancer and head and neck cancers is well established, the involvement of the virus in *BC* is more controversial. [11] Some studies have demonstrated the presence of *HPV high-risk types (16, 18 and 33)* in breast cancer specimens from diverse populations around the world.[12] Women that have both breast and cervical cancers were found to be infected with the same *HPV* types. [16] The controversy surrounding the role of *HPV* in *BC* may be because of the difficulty that has been encountered in detecting the virus in breast specimens in contrast to the relative ease of detection in cervical cancers.[17] Indeed in a previous study from our group, we demonstrated that it was necessary to use *SYBR Green PCR* methods for detection of the virus in *BC* tissues.[17] Because there is a considerable proportion of *BC* specimens are non-cancerous and that the levels of virus are low in *BC*. One solution to the detection of such low levels of *HPV* is the use of *In situ PCR.* The oncogenic mechanisms by which *HPV* induces *BC* have been intensively studied. [18] In this study *HPV* used *was associated with BC* as a model. *HR-HPV* encodes a series of proteins, designated as early *(E1–E7)* or late *(L1 and L2*). Although all of the viral proteins have a role in viral replication, only a small number of the viral early proteins have a role in cellular transformation. Key to transformation are the *(E6 and E7) onco-proteins*, which work in concert to disrupt *cell-cycle regulation* by inhibiting apoptosis and stimulating *cell- cycle* progression by binding/inhibiting the *p53* and *p10 Rb tumor suppressor genes*, respectively. In addition to that the *HPV (E5 and E6)* acts early in transformation (before integration) and are known to disrupt cytokeratin causing perinuclear cytoplasmic clearing and nuclear enlargement which leads to the appearance of a koilocyte. [19]

Justification

- To the best of our knowledge, this is the first study that attempts to determine the possible role of *HPV genotypes* in the pathogenesis of *BC* in Sudan.

Objectives

General objective

The study is to evaluate the possible role of *HPV* in the pathogenesis of *BC* in Sudan.

Specific objectives

- To determine the genotyping of *HPV* in *BC.*
- To find out the relation between *HPV* infections with risk factors such as age, socioeconomic status, family planning, type of tissue, obesity, martial statues in *BC* patient.

Study Design

A retrospective hospital-based case-control study will be conducted.

Study Center

Radiation & Isotopes Center in Khartoum (RICK), in Sudan

Study population

Patients

143 formalin fixed paraffin wax embedded breast tissue blocks were used in this study. Of the (143) breast tissue blocks, (100) were patients' blocks with breast cancer (ascertained as cases) and the remaining (43) were with breast inflammatory conditions ascertained as control).
Tissue blocks were collected from different histopathology laboratories in Khartoum State and other states which mostly received by *RICK* for treatment plan. Full clinical data were obtained from the laboratory information system and reports and general hospital registry office including: age, diagnosis, menopausal status, histological type, grading, age, socio-economic status, marital status, radiation, family history, inflamed breast tissue samples detected at the safety margin of the malignant breast cases was considered as controls the samples diagnosed in institute of endemic disease university of Khartoum labs.

Control

Sudanese women will be included. They will be matched to the patients with respect to their age.

Data Collection

Sources of data will include: clinical examination (done by the physician in the respective hospitals), a questionnaire and laboratory investigations.

Questionnaire

A simple questionnaire is designed and used for each patient; included complete demographic characteristics, present and family history of cancer, personal habits and medical history. Another questionnaire will be designed for control subjects including same questions.

Sample Size

The formula to calculate the sample size is:
n= t^2 x **p (1-p)**
M
Where:
N = required sample size.
t = confidence level at (95%), (standard value of 1.96).
p = estimated prevalence of the disease in the area.
m = margin of error at (5%), (standard value of 0.05).

Sampling Techniques

Specimens

Breast tissues will be collected from Formalin – Fixed, Paraffin - embedded tissues. For the control group tissue inflammatory breast lesion; tissues will be obtained from Formalin – Fixed, Paraffin - embedded tissues. Tissues must be diagnosed as inflamed breast lesions.

Laboratory Tests

1. *DNA* extraction.
2. *PCR* technique.

DNA Extraction procedure

(10)µm sections were cut from the formalin fixed paraffin embedded *BC blocks*, and then pooled in eppendorf tubes. Next the following procedure was used:

1- (1) ml of xylene was added to each tube and the tubes were vortexed vigorously for (10) sec to deparaffinize the tissue. Next, the mixture was centrifuged at full speed for (2) min at room temperature and the supernatant was removed by pipetting while the pellet remained in the tube; this step was repeated two times.
2- (1) ml ethanol (96%-100%) was added to each pellet, mixed by vortexing centrifuged at full speed for (2) min at room temperature and the supernatant was removed by pipetting. This step was repeated two times to remove residual xylene.
3- Next, eppendorf tubes containing the tissue pellets were opened and incubated at (37) °C for (10-15) minutes until the ethanol evaporated.
4- Each pellet was resuspended in (180) µl ATL lysis buffer and (40) µl proteinase K, mixed by vortexing, and incubated at (56) °C in a shaking water bath until the tissue was completely digested.
5- (200) µl AL buffer and (200) µl of ethanol (96%-100%) were added to each tube, and mixed thoroughly by vortexing.
6- The mixture was transferred to a minelute column placed in a collection tube. This assembly was centrifuged at (8000) rpm for (1) min and the flow-through were discarded.
7- Each minelute column was washed two times using the washing solution (AW1 and AW2).
8- Finally, *DNA* was eluted from each minelute column (placed into a new eppendorf tube) using (20-100) µl AE elution buffer by centrifugation at full speed for (1) min. Solution obtained containing the *DNA* was stored at (20) C° for later use.

Selection of Primers

The primers were designed to detect the *HPV types 16, 18.31.33* using the *NCBI* primer design tool[(154)] (Primer sequences and the expected *PCR* products sizes of each pair are available in **(Table 2.3)** *GAPDH (Glyceraldehydes 3-phosphate dehydrogenase)* gene primers were used as controls, to demonstrate the integrity and the quality of the isolated *DNA*.

Table (1.3): Sequences and properties of designed *PCR* primers used to amplify the *HPV16, 18, 31, and 33* used in this study

Primers	Primer sequence(5'-3')	Length	TM(°C)	Product size (bp)
HPV16 Forward Reverse	5-CCACAGGAGCGACCCAGAAAGTT-3 5-ACCGGTCCACCGACCCCTTATAT-3	23 23	61.3 61.7	390
HPV18 Forward Reverse	5-GCGCGCTTTGAGGATCCAACAC-3 5-TGGCACCGCAGGCACCTTAT-3	22 20	61.4 62.2	323
HPV31 Forward Reverse	5-GGCCTCCAAGGAGTAAGACC-3 5-CCCCTCTTCAAGGGGTCTAC-3	20 20	57.2 56.7	157
HPV33 Forward Reverse	5- CAC AGT TAT GCA CAG AGC TGC-3 5- CAA CGA GGT AGA A GA AAG CAT C-3	21 22	61.4 62.2	321

Table (2.3): PCR protocols of amplification product for samples

Primer sequence (5'-3')		Cycling profile	25 µl PCR reaction mixture
HPV16	F: 5-CCACAGGAGCGACCCAGAAAGTT-3 R 5-ACCGGTCCACCGACCCCTTATAT-3	95°C for 5 min 40x 95°C for 45 sec 54°C for 1 min 72°C for 45 sec 72°C for 5 min	12.5 µl master mix 1 µl F.P 1 µl R.P 7.5-9.5 µl NFW 1-3 µl DNA
HPV18	F: 5GCGCGCTTTGAGGATCCAACAC-3 R: 5-TGGCACCGCAGGCACCTTAT-3	95°C for 5 min 40x 95°C for 45 sec 54°C for 1 min 72°C for 45 sec 72°C for 5 min	12.5 µl master mix 1 µl F.P 1 µl R.P 7.5-9.5 µl NFW 1-3 µl DNA
HPV31	5-GGCCTCCAAGGAGTAAGACC-3 5-CCCCTCTTCAAGGGGTCTAC-3	95°C for 5 min 40x 95°C for 1 min 54°C for 1 min 72°C for 1 min 72°C for 5 min	12.5 µl master mix 1 µl F.P 1 µl R.P 7.5-9.5 µl NFW 1-3 µl DNA
HPV33	5- CAC AGT TAT GCA CAG AGC TGC-3 5- CAA CGA GGT AGA AGA AAG CAT C-3	95°C for 5 min 40x 95°C for 1 min 54°C for 1 min 72°C for 1 min 72°C for 5 min	12.5 µl master mix 1 µl F.P 1 µl R.P 7.5-9.5 µl NFW 1-3 µl DNA

Polymerase Chain Reaction *PCR* Genotyping of *HPV*

To amplified (4) types of *HPV (16, 18, 31 and 33)*, specific primers were used to detect them in *BC* tissues (inflammatory as control, cancers as cases). Amplification was performed according to *HPV* kit from (Sacace technologies- Casera –Italy). The *PCR* was carried out in a total reaction volume of (40) µl containing between (20) µl mix-1(contained in PCR tubes), (10) µl of mix-2 and (10) µl of *extracted DNA* (sample). Negative control and positive control of high risk *HPV DNA* tubes contained (10) µl of *DNA buffer*, (10) µl of *high risk HPV DNA*. Samples and controls were amplified using Gene Amp *PCR system (9700)* (reagents and primers provided by Sacace technologies-Casera –Italy). The *PCR* protocol was described in **table (2.3)**

Agarose Gel-electrophoresis

The *PCR* products were visualized in (1.5%) Agarose gel with (0.5) µg/ml Ethidium bromide staining, the gel was prepared by dissolving (0.7) gm of agarose powder in (35) ml of 1X TBE buffer and heated at (65)°C until the agarose completely dissolved, then left to cool at room temperature and (2) µl Ethidium bromides was added. The comb was then placed appropriately in the electrophoresis tray and then gel was slowly poured and left to set for (30) min for solidification. In a clean Eppendorf tube (10) µl of (1000) bp *DNA ladder* and *PCR* product was loaded on the gel. Gel-electrophoresis was performed at (120V) and (36 Am) for (60) minutes. Pictures were taken by Gel documentation system (Gel mega, digital camera and software in a computer).

Interpretation of PCR results

According to manufacture *high risk HPV* kit (from *Sacace technologies- Casera –Italy*) manual, the *PCR* product length for (*HPV16*) should be (457) bp, *HPV 18* should be (322) bp, *HPV 31* should be (452) bp and *HPV 33* should be (398) bp to be visualized after staining with ethidium bromide.

Ethical Considerations

Ethical clearance to conduct this study has been obtained from the Ethics Committee of the Ministry of health, Khartoum state; Initial consent will be obtained from authorities of the study areas.

Data Analysis

1. Deceptive analysis type
2. Analytical data analyses are used in this study.

The data will be cleaned and checked for consistency before entering it for analysis. Analysis will be conducted using Epi Info Version (6) (The Epi Info computer programs produced by *CDC* and the World Health Organization. *(WHO)* provide public-domain software for word processing, database management, and statistics work in public health) the alpha (α) level of significance will be set at (0.05). Descriptive analysis will be done by using ***(Chi square test)***.

Results

Table (1.4) Distribution of *HPV* genotypes in BC patients

PCR result of HPV	No of positive Sample	%
HPV genotype 16	22	33%
HPV genotype 18	21	31%
HPV genotype 31	10	15%
HPV genotype 33	14	21%
Total of Sample	67	100%

The above table shows that HPV genotype 16 is most prevalent in breast cancer specimens

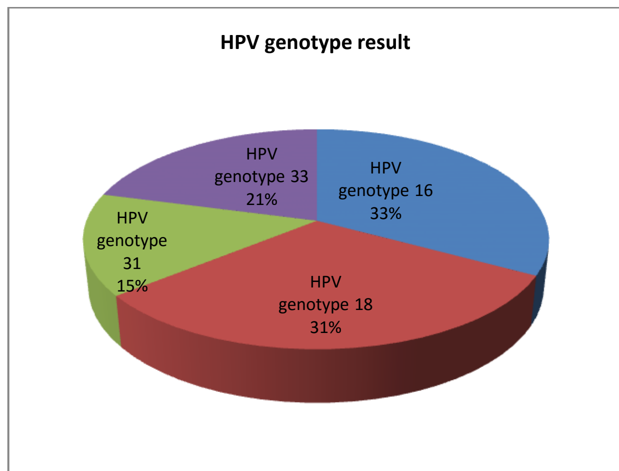

Figure (1.4) Description of *HPV* infection with genotypes

Table (2.4) Distributions of HPV genotypes with different histopathological types of BC

Type of breast cancer	*HPV* genotype			
	16	18	31	33
Invasive ductal carcinoma	13(54%)	9(45%)	0(0%)	2(14%)
Invasive lobular carcinoma	5(25%)	6(30%)	0(0%)	2(14%)
invasive micropapillary carcinoma	0(0%)	0(0%)	(0%)0	9(65%)
Medullary carcinoma	5(25%)	5(25%)	(0%)0	1(7%)
Inflammatory breast Sample	0	0	10(100%)	0

In this table HPV genotype 16, 18 are more dominant in breast cancer specimen when compares with inflammatory breast conditions as control group **table (3.4).**

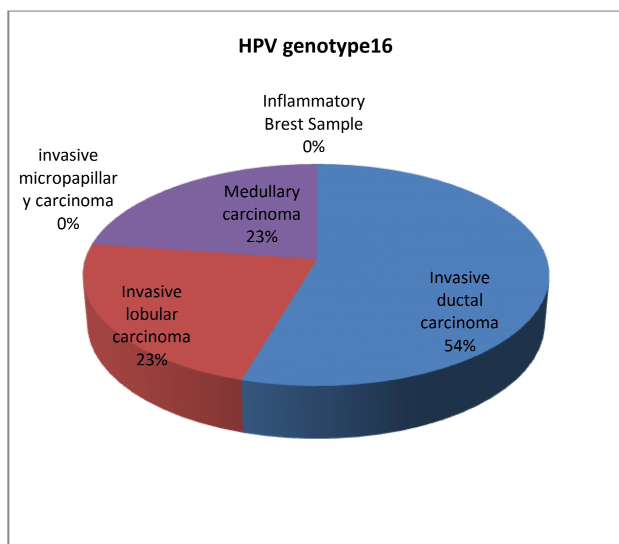

Figure (2.4) Description of *BC* types by *HPV* genotype 16

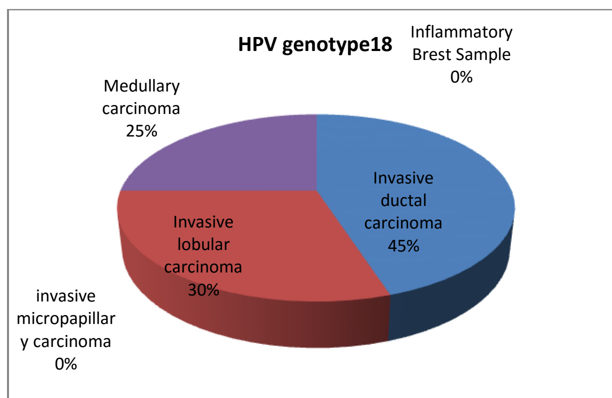

Figure (3.4) Description of *BC* types by *(HPV)* genotype 18

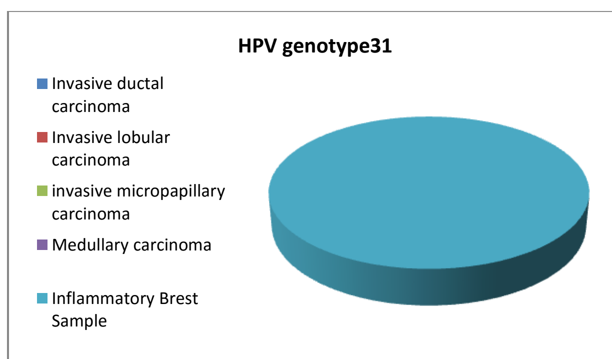

Figure (4.4) Description of *BC* type by *HPV* genotypes 31

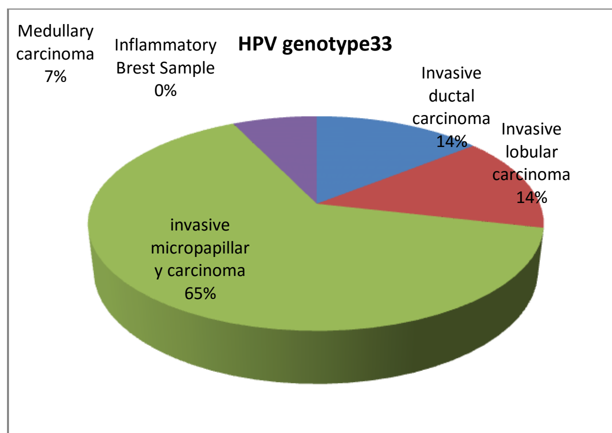

Figure (5.4) Description of *BC* type by *HPV* genotypes 33

Figure (6.4): Separation of the GAPDH PCR product by (1.5%) agarose gel electrophoresis Lane

1. 100 bp ladder. Lane
2. PCR negative control. Lanes
3-9: 157 bp amplified *GAPDH* product in 7 breast samples.

Figure (27.4) Separation of *PCR* product for *HPV* by (1.5%) agarose gel electrophoresis lane

1. 100 bp ladder lane.
2. *PCR* negative control lane.
3. Positive control for *HPV* 18 lanes.
4-10: breast cancer samples were negative for *HPV* 18**.**

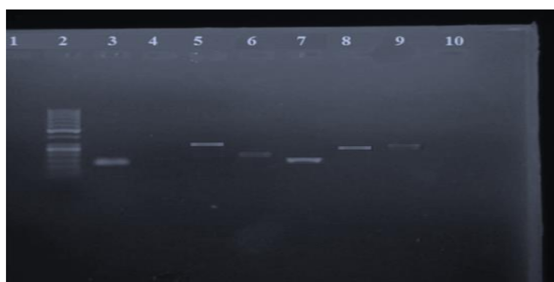

Figure (30.4): Separation of *PCR* products *HPV genotypes* to control and samples by 1.5% agarose gel electrophoresis Lane

1. Ng Control.
2. 100 bp *DNA* marker.
3. *HPV* positive sample 267 bp.
4. *HPV* negative sample.
5. *HPV* genotype 16 (457 bp).
6. *HPV* genotype 18 (322 bp).
7. *HPV* genotype 31 (263 bp).
8. *HPV* genotype 33 (387 bp).
9. *HPV* genotype 16 (457 bp).

Discussion and Recommendations

Discussion

Breast cancer is the most common among females and comprises about (18%) of all cancers affecting them. About (1.7) million new cases are reported in the world each year. Based on the most global recent data, approximately (12.3%) of women are diagnosed with *BC* at a point of time during their life.[12, 15] However, so far and to the best of knowledge there are no records regarding the prevalence and incidence of breast cancer cases in the Sudan.

In this study, (143) cases were screened for breast pathology, (100) were diagnosed to have breast cancer, the remaining (43) diagnosed as having inflammation of the breast (control).

Of the (100) cases, (41) patients (29%) were diagnosed with invasive ductal carcinoma, 21(15%) with invasive lobular carcinoma, 12(8%) with invasive micropapillary carcinoma and finally, 26(18%) with medullary carcinoma **table (1.4).**

Genotyping of the infected *PCR-positive HPV* specimens was performed. Out of the (143) specimens (*BC* and breast inflammation) the following genotypes were determined and mentioned in a descending order. Twenty two (33%) of the *HPVs* were of the genotype 16, 21(31%) genotype 18, 14(21%) genotype 33 and 10(15%) genotype 31 **table (2.4).**

The study findings are similar to those reported in a study from Nigeria[78, 90] in which *HPV genotype*16 was the most common genotype in breast cancers and [76, 77] reported that, *HPV*16, 18, 33 respectively are frequently detectable in high grade of breast carcinoma,

These results are different from a study conducted in Australia which reported genotype (18) to be the most prevalent and had an affinity or tropism to glandular as compared with squamous epithelial cells. [33, 35] This difference may be due to the variation in the environment and the social habits.

The other oncogenic *HPV types* (18 and 33) are also detected in invasive cervical cancer biopsies were also detected in breast biopsies. [85, 68] Another study conducted in France by [56, 57] they assessed *HPV genotypes* distribution in breast cancers and they found (*HPV genotypes* 16) was the most prevalent types it was founded in (89.7%) of *HPV-positive* breast cancer cases which was similar to our findings the study by. ([56, 34] in Sweden also showed to the presence of *HR-HPV* in breast cancers particularly type (35).

Also this study finding is similar to other study by. [67.77] where they determine the role of *high-risk HPV infections* in human *BC* in Middle Eastern women, they investigated the presence of *high-risk HPV types* 16, 18, 31, 33 and 35 in a cohort of (113) breast cancer samples from Syrian women by *PCR* , they found that 69 (61.06%) of the (113) samples are *HPV positive* and 24 (34.78%) of these specimens are co-infected with more than one *HPV genotypes* and they found that *HPV types* 16, 18 and 31 are present in only 10, 11 and 8 cancer tissues respectively.

Several studies failed to detect HPV in breast carcinoma such as the study by[45, 56] were they studied (HPV genotypes 11, 13, 16, 18, 30, 31, 32, 33, 45, 51) in (95) women with breast cancer without detecting any of these subtypes this failed is me by due to the sample size were they used.

Another study by [45, 67] were they founded *HPV genotypes* 16, 18 in 13 *(IDC)*, 15 micropapillary carcinomas cases, furthermore the [45.46] used six different primers, including the following genotypes 16, 18, 31, 33,

45 in (81) Swiss women cases with breast cancer they founded no *positivity of HPV* was detected in other study such as the study by. (67,56) where they studied the prevalence of *HPV genotypes 16, 18, 33, and 45* and low risks (6, 11) in (50) breast cancers in women in France by other studies have demonstrated the presence of *HPV high-risk types 16, 18* and 33 in breast cancer specimens from diverse populations around the world: Italy, Norway, China, Japan, USA, Austria, Brazil, Australia, Taiwan, Turkey, Greece, Korea, Mexico, Hungary and Syria (45, 46, 68, 78, 79, and 82) the prevalence of *HPV* positive breast cancer in these studies was reported to vary from (4%) in Mexican to (86%) in American women.

Recommendations

1- Further advanced studies, in general, are required to understand the pathology of *HPV* as very few have been performed in both the Arab and African worlds.

2- To develop new methodology for the diagnosis and identification of *HPV* in *HPV-affected tissues* and in particular those from the breast.

3- More studies are strongly needed to shed light on the mode(s) of infection of *HPV* as no clear information is available regarding this.

4- To nip in the bud any development of *BC*, primary and secondary school girls are to be trained for self-investigation of any breast tumors.

5- Research for the possible development of a vaccine against *HPV* is long-awaited.

6- To advance the frontiers of treatment, funds are urgently needed for the provision of the materials and equipment necessary to serve both levels of routine diagnosis as well as more research on *HPV*.

7- The isolation of *HPV* from affected tissue(s) in order to understand its behavior and its pathogenesis.

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