# Molecular Study of P53- and Rb-Tumor Suppressor Genes in Human Papilloma Virus-Infected Breast Cancers

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## Abstract

The study was aimd to define the percentage of detection of high- oncogenic risk types of HPV and their genotyping in archival tissue specimens that ranged from apparently health tissue to invasive breast cancer by using one of the recent versions of In Situ hybridization(ISH) .2.To find out rational significance of such genotypes as well as over expressed products of mutants P53 and RB genes on the severity of underlying breast cancers .

The DNA of HPV was detected in 46.5 % of tissues from breast cancers while HPV DNA in the tissues from benign breast tumors was detected in 12.5%. No HPV positive – ISH reaction was detected in healthy breast tissues of the control group. HPV DNA of genotypes (16, 18, 31 and 33) were detected in malignant group in frequency of 25.6%, 27.1%, 30.2% and 12.4%, respectively. Over expression of p53 was detected by IHC in 51.2% breast cancer cases and in 50% benign breast tumor group , while none of control group showed P53- over expression . Retinoblastoma protein was detected by IHC test in 49.7% of malignant breast tumors, 54.2% of benign breast tumors but no signal was reported in the tissues of control group .

**Key words :** HPV , breast tumors , p53 , Rb , ISH , IHC

## الخلاصة

تم إعداد الجانب العملي لهذه الدراسة فاتخذ مسارين رئيسين:-

1. تحديد وجود الحامض النووي الدنا (DNA) للفايروس الحليمي البشري ذوانماط الاختطار السرطاني العالي 31,18,16و33 بفحصها داخل خلايا الأنسجة المختلفة حيث تم انجازه بوساطة استخدام تقنية التهجين الموضعي ذات الحساسية العالية جدا .
2. التحري عن انتشارالتعبيرالفائق للبروتينات المشفره من الجينات المثبطه للاورام, P53 والريتونابلاستوما(RB).

شكلت مجموعةِ العينات المحفوظة لسرطانات الثدي والتي أظهرت نتائج موجبة للفيروس الحليمي البشري نسبة( 46.5% ) من مجموع هذه العينات بينما وجدت نتائج موجبة للفيروس الحليمي البشري بنسبة (12.5%) في مجموعة ورم الثدي الحميد.كانت نسب الاصابات بالفيروس الحليمي البشري ذوالانماط 31,18,16و33 في مرضى سرطان الثدي 25.6%, 27.1%,30.2%و12.4%,على التوالي.تم تحديدالنسب المئويه للحالات الموجبه لانتشارالتعبيرالفائق للبروتين المشفر من الجين المثبط للاورام (P53) والتي حددت بالاختبارالكيميائي النسيجي المناعي في عينات نسيج سرطان الثدي وورم الثدي الحميد(51.2% و50% )على التوالي.بينما لم تسجل أي حاله موجبه في انسجة الثدي السليمه .تم تحديدالنسبة المئويه للحالات الموجبه لانتشارالتعبيرالبروتيني من الجين المثبط للاورام الرتينوبلاستوما (Rb)والتي حددت بوساطة الاختبارالكيميائي النسجي المناعي في عينات نسيج سرطان الثدي وورم الثدي الحميد(49.7% و54.2% )على التوالي.بينما لم تسجل أي حاله موجبه في انسجة الثدي السليمه .

**الكلمات المفتاحية:** الفايروس الحليمي البشري، سرطان الثدي، تقنية التهجين الموقعي، بي 53، ريتونا بلاستوما.

## Introduction

Although many attempts have been made , yet, successful tissue culture system for propagation of papillomaviruses has not been developed . Therefore , human papilloma viruses had been characterized by molecular hybridization and recently phylogenetic relationships based on nucleotide and amino acids sequence alignment had gained wider acceptance and replaced the classic phenotypic classification (Herrington *et al.*, 1999; Henning *et al.*, 2009) .

Mucosal HPV are small double-stranded DNA viruses that infect mainly anogenital epithelium (Christos *et al.,* 2006) .

The majority of these sexually transmitted genital HPV types are considered high risk because they possess at least three proteins E5 , E6 and E7 with growth-stimulating and transforming properties (Zur Hausen , 2006) .

Breast cancer (BC) is a heterogeneous category of tumors, characterized by different classes of gene expression profiles (Elisabet *et al.,* 2011). A positive family history or genetic factor has been confirmed to be a major contributor to the risk of developing this disease , and this link is particularly important for early-onset breast cancers (Yong *et al.,* 2010) .

In recent years , evidence has emerged which indicates that HPVs may also have a role in breast cancer . HPV high risk types 16 , 18 and 33 have been identified in BC from widely different populations (Lawson *et al.,* 2010) .

The BC is the most common cancer observed in patients with the Li_Fraunmeni Syndrom(LFS) inheriting the analogous P53 mutations (Olivier *et al.,* 2003) .

P53 tumor suppressor gene that controls cellular growth and differentiation accounts for the majority of families fulfilling classical criteria for LFS and for 40% of families with the less stringent criteria of Li_Fraumeni_like Syndrom (LFL), which are autosomal dominantly inherited disorders characterized by the occurrence of early – onset BC , Sarcomas and other neoplasms (Yong *et al.,* 2010 ) .

The P53 gene can be inactivated by somatic (sporadic) and less commonly, germ line (inherited) mutation. Among transforming proteins of several DNA viruses, E6 protein of HPV can bind and degrade P53 . The Mechanism for this effect appear to include inhibition of the normal suppressor function (Wang, 1993; Awson *et al.,* 2009) .

The retinoblastoma (RB) protein plays a key role in regulating cell cycle. The RB gene has been found mutated in several types of cancers where germ line mutation in the RB gene lead to retionblastomas (Elisabet *et al.,* 2011). The RB gene was the first tumor suppressor to be cloned, but the mechanism behind it is role in tumors remains unclear (Marie and Harlow, 2002) .

Inactivation of the RB gene in BC was originally shown using a series of cell lines (Jason *et al.,* 2008) . 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 (Krawczyk *et al.,* 2008) . Key to transformation are the E6 and E7 oncoproteins, which work to disrupt cell-cycle regulation , inhibit apoptosis and stimulate cell-cycle progression by binding inhibiting the P53 and RB p110 tumor suppressor genes, respectively. In addition, HPV E5 and E6 act early in transformation (before integration) and are known to disrupt cytokeratin causing perinuclear cytoplasmic clearing and nuclear enlargement which leads to the appearance of koilocytes (Thmison *et al.,* 2008 ; Heng *et al.,* 2009) .

## Material and Methods

The study was designed as a retrospective one. It has recruited 173 selected formalin fixed, paraffin embedded breast tissue blocks (123 breast carcinomas, 24 benign epithelial breast tumors and 20 blocks from normal breast tissues as a control group. The age of patients ranged from 16–72 years. The specimens were collected during the period from November 2008 to April 2012. from Al-sader Teaching Hospital, Hilla Teaching Hospital, Dr. Assad AL- Janabi private Laboratory, Dr. Mazin private Laboratory, Dr. AL-Mohessin private Laboratory, Dr. Ali Zaki private Laboratory and Dr. Hadi AL-Mousawy private Laboratory. The diagnosis of these tissue blocks were based on the obtained pathological records of these cases from

hospital files as well as histopthological laboratories records. Four $\mu$ m thick sections were made and sticked on positively charged slides. In situ hybridization/detection system (Zytovision GmbH. Bremerhaven. Germany) was used to target DNA sequences in tissue specimens using Digoxigenin–labelled cocktailed HPV DNA probes for wide range of high risk HPV genotype including16/18/31/33/35/45/51/82. In situ hybridization / detection system (Maxim Biotech Inc. USA) used to target DNA sequences using biotinylated long DNA probe for HPV 16, 18, 31 and 33in tissue specimens. Methods were conducted according to the instructions of manufacturing company. Positive control reactions were performed by replacing the probe with biotinylated house keeping gene probe. For the negative control, all reagents were added except the diluted probe. Proper use of this hybridization/ detection system gave an intense blue signal at specific sites of the hybridization probe in positive test tissues. The signal was evaluated under light microscopy using x 100 lens for counting the positive cells. ISH was given percentage scores based on positive signals and number of cells that gave these signals. 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 assigning cases to one of the three following percentage score categories : score (1) = 1–25%, score (2)= 26 – 50%, score (3) = >50% ( 10).

Immunohistochemistry / Detection system (Us Biological Inc . USA) was used to demonstrate the p53 &Rb tumor suppressor genes . This technique is based on the detection of the product of gene expression (protein) in malignant and normal cells using apecific monoclonal antibodies , i.e. Primary antibody for specific epitope (usually mouse antihuman monoclonal antibody ) , which binds to nuclear targeted protein . The bound primary antibody is then detected by secondary antibody (usually rabbit or goat anti mouse), which contains specific label (in this context we used peroxidase labeled polymer conjugated to goat anti mouse immunoglobulin). The substrate is DAB in chromogen solution , positive reaction will result in a browning color precipitate at tne antigen site in tested tissues ( EL – Sisy , 1999).

Chi –square test was used to detect the significance between variables of our study . All the statistical analysis was done by SPSS program(Version– 17)&P value was considered significant when $p < 0.05$.

## Results

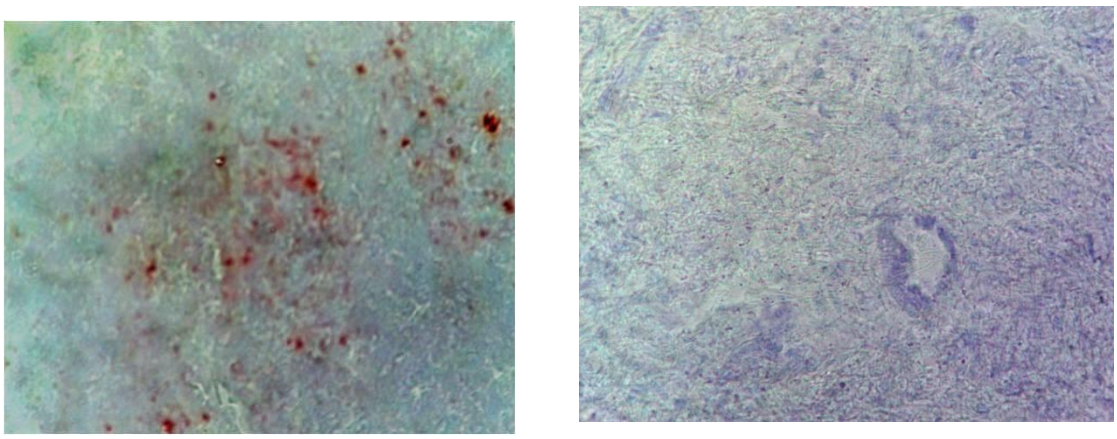
### Detection and Genotyping of HPV

The signals of ISH were detected as red discoloration at the site of sequence-complementarity as nuclear signals (Table 1 and Figure 1).

**Table (1): Frequency distribution of HPV DNA signal scoring among the malignant breast cancers , benign breast tumors and healthy breast tissues.**

| HPV signal scoring | | Breast Malignancy (n=129) | | Benign breast tumor (n=24) | | Normal Breast Tissue (n=20) | | P |
|---|---|---|---|---|---|---|---|---|
| | | N | % | N | % | N | % | |
| Negative | | 69/129 | 53.5 | 21 | 87.5 | 20 | 100.0 | 0.001 significant |
| Positive | | 60/129 | 46.5 | 3 | 12.5 | 0 | 0.00 | |
| Scoring | I | 9/60 | 15.0 | 0 | 0.0 | 0 | 0 | |
| | II | 20/60 | 33.3 | 1/3 | 33.3 | 0 | 0 | |
| | III | 31/60 | 51.7 | 2/3 | 66.7 | 0 | 0 | |
| Mean Rank | | 95.6 | | 67.1 | | 55.5 | | |

**Table (1)** shows the positive results of HPV DNA-ISH detection ,where 46.5% ( 60 of total 129) malignant breast cancer cases showed positive signals ,whereas the benign group revealed 12.5% positive signals that represented 3 out of 24 cases of this group. None of control group presented positive signal for HPV-ISH test. In the present study, the highest percentage of HPV score signaling (51.7%: 31 out of 60 cases) was found to have high score(III),while in the benign tumors group it was found that( 66.7%2 : out of 24 cases) have such high score. Statistically, highly significant differences ( $p < 0.05$) were found on comparing the results of these study groups .



**A** **B**

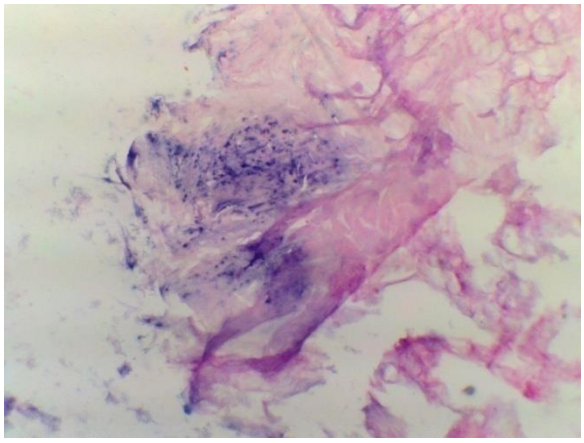
**Figure(1) :**In Situ Hybridization(ISH) for Generic HPV Deduction Infiltrative Breast Cancers Using Digoxigenin-Labeled HPV (Cocktailed) Probes ;Stained with 3-Amino 9-Ethyl Carbazole (Red) and Counter Stained by Nuclear Blue Solution(Blue). A.Breast Cancer with negative HPV –ISH reactions (40X) .B.Positive HPV-ISH reaction with strong score and high signal intensity (40X).

The signals of ISH were detected as blue discoloration at the site of sequence-complementarity as nuclear signals ( Table 2 & Figure 2) .

**Table (2) : Percentage of Different HPV Genotypes in Malignant Breast Tumors Group .**

| Probe -ISH positivity ( n=129) | Total positive | | Score signal | | | | | | 95% confidence interval |
|---|---|---|---|---|---|---|---|---|---|
| | N | % | I | | II | | III | | |
| | | | N | % | N | % | N | % | |
| HPV genotype 16 (n=60) | 33 | 25.6 | 13 | 39.3 | 6 | 18.1 | 14 | 42.4 | (18.1 – 33.1) |
| HPV genotype 18 (n=60) | 35 | 27.1 | 14 | 40.0 | 12 | 34.3 | 9 | 25.7 | ( 19.4 – 34.8) |
| HPV genotype 31 (n=60) | 39 | 30.2 | 19 | 48.7 | 13 | 33.3 | 7 | 35.0 | ( 22.3 – 38.1) |
| HPV genotype 33 (n=60) | 16 | 12.4 | 7 | 43.7 | 8 | 50.0 | 1 | 6.3 | ( 6.7 – 18.1) |

HPV DNA of type ( 16 , 18 , 31 and 33) were found in malignant group in 33 (25.6%) , 35 ( 27.1%) , 39 ( 30.2%) and 16 ( 12.4%) cases, respectively . Significant correlation ( $p < 0.05$) of all genotypes of HPV were found among study groups .



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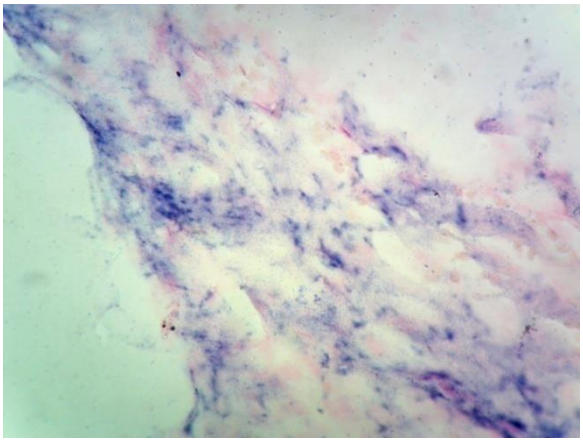

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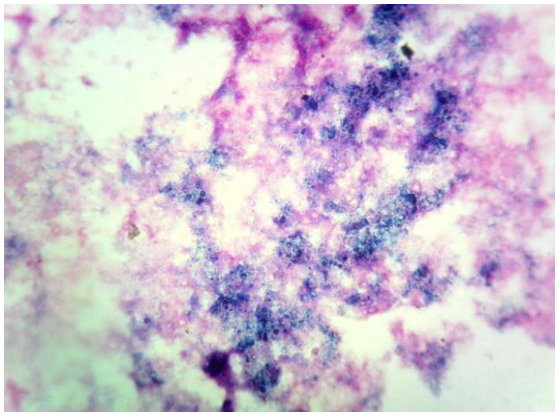

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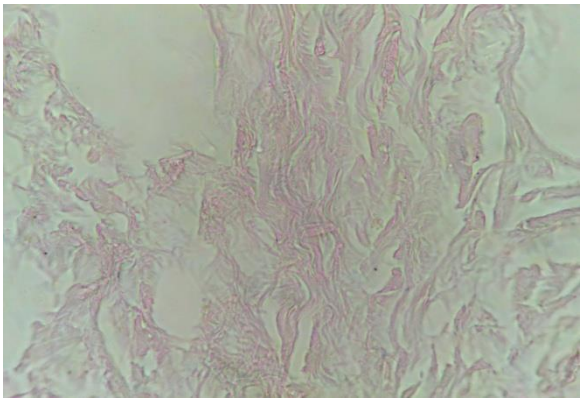

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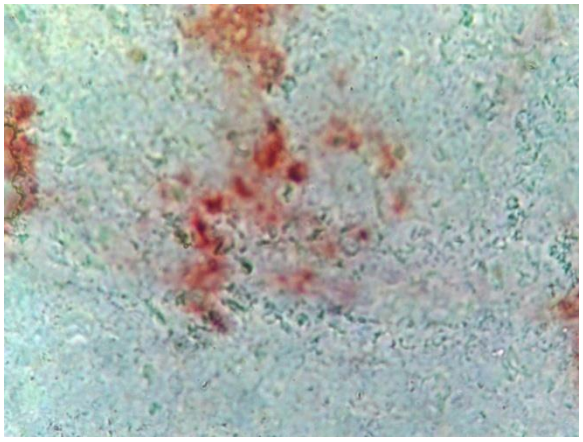

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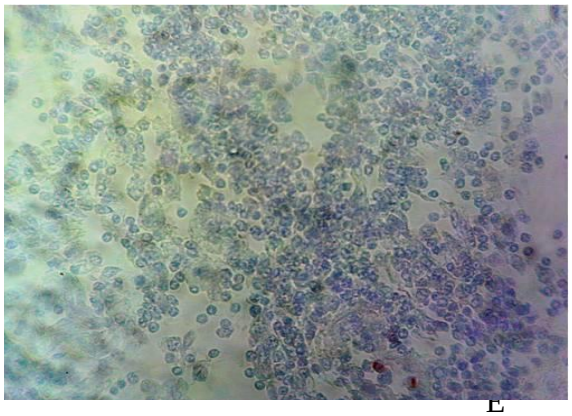


E

**Figure(2) :In Situ Hybridization(ISH) for HPV-16 Deduction Infiltrative Breast Cancers Using Biotinylated -Labeled HPV-16 Probe;Stained with NBT/ BCIP (Blue)and Counter Stained by Nuclear Fast Red (Red).**

**A. Breast Cancer (HPV 16-positive) ; B. Breast Cancer (HPV 18-positive); C. Breast Cancer (HPV 31-positive); D. Healthy breast tissue (HPV-negative); E. Breast Cancer (HPV 33-positive); F-Healthy breast tissue (HPV-negative).**

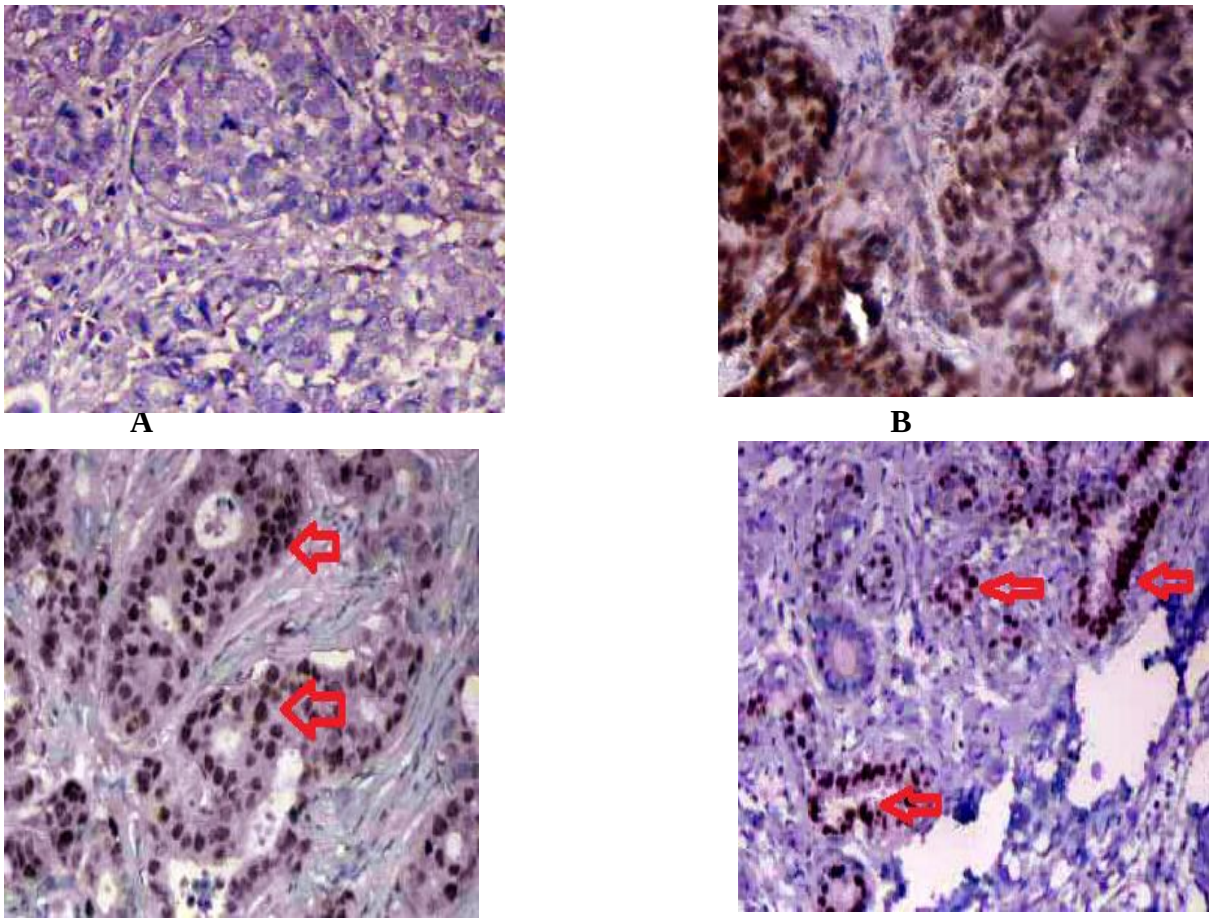
## Tumor Suppressor Genes

### Immunohistochemistry For P53

In the current study ,positive p53 immunohistochemistry nuclear staining was detected in 51.2% of malignant breast tumors while in benign breast tumors was detected in 50%. None of control group showed P53- over expression(Table 4-14).

**Table(3): Frequency distribution of immunohistochemistry results of P53 protein according to the signal scoring.**

| P53 over expression | | Healthy breast tissues (n=20) | | Benign breast tumors (n=24) | | Breast Cancers (n=129) | | P |
|---|---|---|---|---|---|---|---|---|
| | | N | % | N | % | N | % | |
| Negative | | 20/20 | 100.0 | 12/24 | 50.0 | 63/129 | 48.8 | < 0.001 significant |
| Positive | | 0 | 0.00 | 12/24 | 50.0 | 66/129 | 51.2 | |
| Scoring | I | 0 | 0.0 | 5/12 | 41.7 | 17/66 | 25.7 | |
| | II | 0 | 0.0 | 5/12 | 41.7 | 27/66 | 40.9 | |
| | III | 0 | 0.0 | 2/12 | 16.6 | 22/66 | 33.4 | |
| Mean Rank | | 100.1 | | 85.1 | | 91.5 | | |



**A** **B**

**C** **D**

**Figure (3): Infiltrative Ductal Carcinoma Showing The Results Of Immunohistochemical Staining Of P53 Protein Over Expression Using Biotinylated -Labeled Anti-P53 Protein Antibody, Stained By DAB-Chromogen (Brown) and Counter Stained By Mayer's Hematoxyline (Blue).A.Breast Cancer with negative P53 –ICH reactions(40X).B. Positive P53 –ICH reaction with strong score and high signal intensity (40X).C. Positive P53 –ICH reaction with moderate score and high signal intensity (40X). D. Positive P53 –ICH reaction with low score and high signal intensity (40X).**

## RB- IHC Signal Scoring

Retinoblastoma protein was detected by IHC test in 49.7% of malignant breast tumors, 54.2% of benign breast tumors but no signal was reported in the tissues of control group.

**Table (4) : Frequency distribution of Immunohistochemistry for RB according to signal score among study groups .**

| RB Protein Immunohistochemistrey | | Healthy breast tissues (n=20) | | Benign breast tumors (n=24) | | Breast Cancers (n=129) | | P |
|---|---|---|---|---|---|---|---|---|
| | | N | % | N | % | N | % | |
| Negative | | 20/20 | 100.0 | 11/24 | 45.8 | 65/129 | 50.4 | < 0.001 significant |
| Positive | | 0 | 0.00 | 13/24 | 44.2 | 64/129 | 49.6 | |
| Scoring | I | 0 | 0.0 | 5/13 | 38.5 | 24/64 | 37.5 | |
| | II | 0 | 0.0 | 5/13 | 38.5 | 22/64 | 34.4 | |
| | III | 0 | 0.0 | 3/13 | 23.0 | 18/64 | 28.1 | |
| Mean Rank | | 48.5 | | 94.7 | | 91.5 | | |

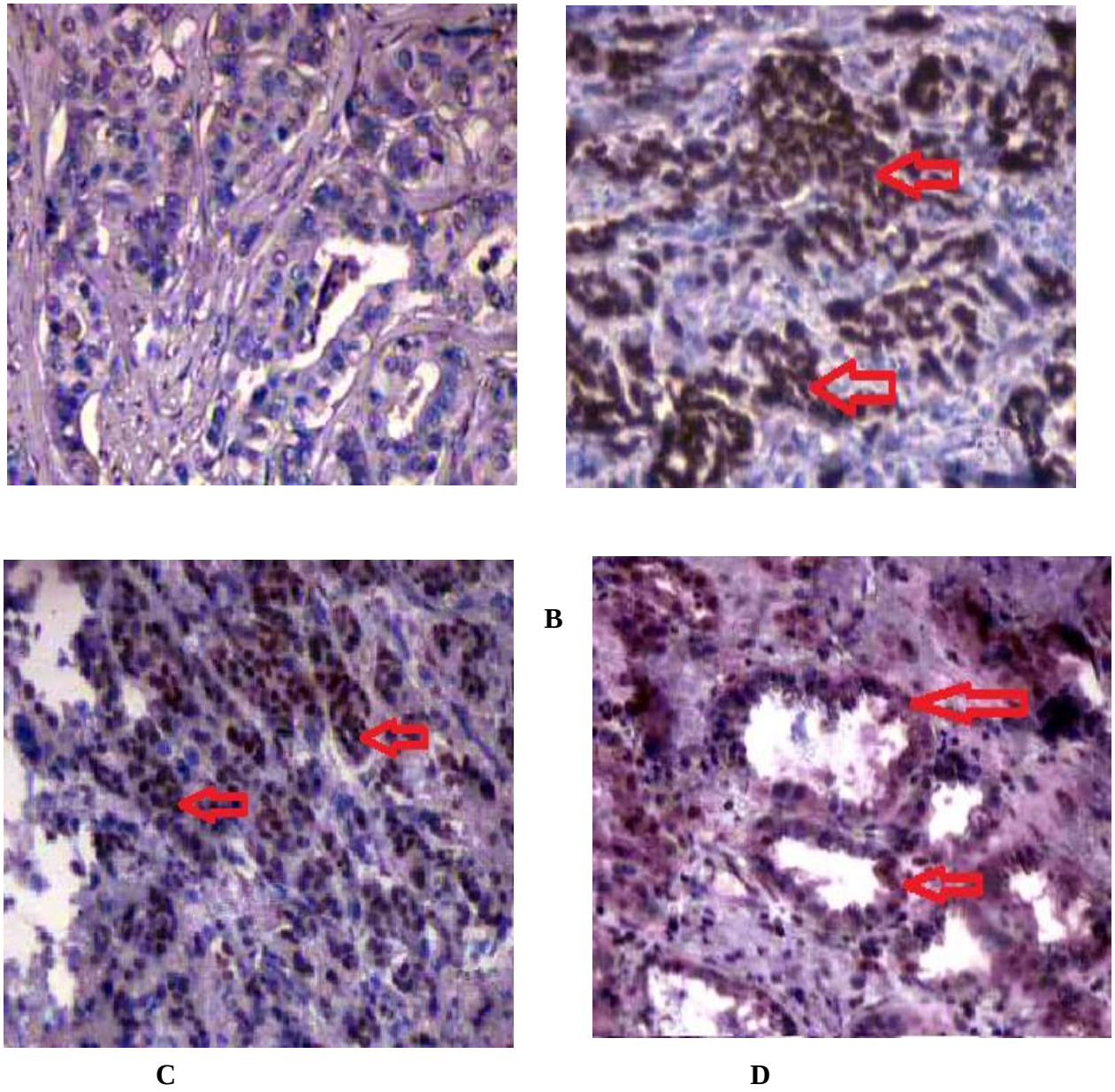


**Figure (4): Infiltrative Ductal Carcinoma Showing The Results Of Immunohistochemical Staining Of RbProtein Expression Using** Biotinylated **- Labeled Anti-Rb Protein Antibody, Stained By DAB-Chromogen (Brown) and Counter Stained By Mayer's Hematoxyline (Blue). A. Breast Cancer with negative Rb –ICH reactions(40X).B .Positive Rb –ICH reaction with strong score and high signal intensity (40X). C .Positive Rb –ICH reaction with moderate score and high signal intensity (40X). D. Positive Rb –ICH reaction with low score and high signal intensity (40X).**

## Discussion

The majority of molecular events in the genesis of breast cancer are unknown . However, initial studies have reported an association of breast cancers with cervical intraepithelial neoplasia III (CIN III) –like lesions (Liu *et al.*,2001; David *et al.*,2008).

High oncogenic – risk HPV genotypes such as HPV-16, -18, -31 and -33, were detected in cases of breast cancers in this study . Despite great variability in the HPV detection rates worldwide ,the majority of HPV types that have detected were the high oncogenic risk- types ( HPV-16 and -18) (Kan *et al.*,2005). The association of high oncogenic-risk HPVs was reported to be stronger in invasive breast carcinomas (Yasmeen *et al.*,2007).

The Transmission route of HPV detected in breast cancer is yet unclear. Two independent studies suggested a possible hematogenic and/or lymphatic transfer of the virus from one organ to another (Widschwendter *et al.*,2004).

De Villiers *et al.*,2005 showed HPV presence in the nipple , suggesting HPV transfer in a retrograde fashion from the nipple via areola , lactiferous ducts and sinuses .

In the present study, the detection of generic / cocktailed high oncogenic risk types was found in 46.5% where the 95% confidence interval was (37.9% - 55.1%).

The reported prevalence of HPV infection in breast cancer shows a great variation worldwide, ranging from O to 86% (Choi *et al.*,2007 , Lindle *et al.*,2007). Demographic features and genetic background may contribute to the geographical difference of HPV prevalence in breast carcinoma world wide .In addition , the difference in published reports may be attributed to the numbers of samples tested methodological difference , and sensitivity of methods used , such as use of different primer sets (Khan *et al.*,2008).

The role of HPV in breast cancer development is not elucidated as in other studies , the presence of HPV sequences in breast tumor samples is not associated with tumor grade , patient mortality , expression of ER,PR, ERB-2 , P53 expression and mutation (Kan *et al.*,2005). In addition , in two independent studies , HPV 16 has been found to be present in breast tumors that occur in European women with HPV-16 associated cervical cancer (Hennig *et al.*,1999 , Widschwendter *et al.*,2004).

The most important negative regulator of cell-cycle progression is the tumor suppressor gene TP53,which has been recorded in about 20-40% of human cancer (Gordon ,2003;Psyrri *et al.*,2007).

The p53 mutation rates in breast cancer vary from 15% to 71% depending on the population (Harmann *et al*.,1997),and the present results are among this range of detection of p53 mutations.

The wild-type p53 protein has a very short half-life and is detected in low levels by IHC. In various studies, cases with wild-type p53 sequence showed over expression of p53 protein (Psyrri *et al.*, 2007).The accumulation and stabilization of normal p53 protein may be caused by non-mutational events (Prives and Hall, 1999).

Theoretically, one should be able to distinguish among p53-negative IHC (null mutant), p53-low (wild type) and p53-overexpressing (non-null mutant) tumors; Therefore, some investigators find that low p53 is a good prognostic marker while others report that low p53 is a poor prognostic marker, depending on the percentages

of null mutants and wild-type p53 in the low p53 category( Psyrri *et al*., 2007). It used to be known that mutant p53 protein has the ability to form a tetramer with wild type p53, acting as a dominant negative to repress normal physiological processes of p53, possibly by inducing an inactive conformation of the DNA binding domain and reducing the ability to transactivate/repress target genes(Chene, 1998; David *et al*.,2012). The RB tumor suppressor is functionally inactivated in a large fraction of human cancers (Emily *et al* .,2007). Structural abnormalities of the RB including chromosal loss and mutation gene have been reported in approximately 20 – 30 % of breast cancers. Besides chromosomal loss and mutation , there are various other mechanisms for RB inactivation. Also ,RB can be inactivated in tumors by the loss of one allele and hypermethylation of the other alleles(Strzaker *et al*.,1997; Foster *et al*., 1998). Interestingly, a recent survey of RB status in metastatic breast cancer revealed two cases with duplication of the entire gene(Berge *et al*.,2011). This may be related to a phenomena observed in colorectal carcinoma, where high expression of pRb was shown ,paradoxically ,to protect from E2F-induced apoptosis (Bernards ,2008; Berge *et al*.,2011).In line with this ,expression of constitutively active phosp-mutant Rb transgenes in mouse mammary epithelium induces adenocarcinoma(Jiang *et al.*,2011). Thus, both activation and inactivation of pRb can be oncogenic in the mammary gland (Jiang *et al.*,2011).

RB inactivation was observed to increase the proliferative potential of the cells which was associated with overexpression of cyclin dependent kinase (Connor *et al*.,2001). The deregulation of the Rb pathway is the primary function of each of the DNA tumor virus oncoproteins that promote cellular proliferation ,this includes the adenovirus E1 A protein, polyoma virus ,SV40 T antigen and HPV E7 protein(Joseph and Nevins ,2001).

## Conclusions

The significance prevalence of expression of mutated p53 & Rb genes as well as high oncogenic risk HPV genotypes in breast cancers could indicate for an important role of these molecular and viral factors in breast carcinogenesis.

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