# INVESTIGATION OF THE ROLE OF VACCINIA VIRUS VH1-RELATED (VHR) IN PROGRESSION OF CERVICAL CARCINOMA

By
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## ABSTRACT:

**Background:** Vaccinia virus VH1-related (VHR) dual-specific protein phosphatase, encoded by the *DUSP3* gene, has been reported to play a critical role in cell cycle progression. Recent studies have demonstrated that lack of VHR expression by cells causes their arrest in G1 and G2 phases of the cell cycle also they showed early signs of cell senescence. The usefulness of $P16^{INK4A}$ in the diagnosis of cases of cervical neoplasia, has been reported in several studies.

**Objectives:** The current study has been designed to assess the role of VHR in progression of cervical carcinoma and to evaluate the significance of $P16^{INK4A}$ as a useful diagnostic biomarker; then to find any correlation between the two examined markers.

**Materials and Methods:** In the present study, we evaluated the expression levels of VHR protein and $P16^{INK4A}$ using Immunohistochemistry in a series of archival formalin-fixed and paraffin-embedded tissue specimens of normal excocervix (n=10), low-grade SIL (n=12), high-grade SIL (n=18) and invasive squamous cell carcinoma (n=20).

**Results:** VHR was expressed at very low level in the cytoplasm of parabasal cell layers of the normal cervical epithelium. VHR was significantly up-regulated in squamous intraepithelial lesions (SILs) of the cervix in LSIL ($p<0.05$) and HSIL ($p<0.001$) in comparison to normal exocervix. In cases of invasive squamous cell carcinoma, VHR was highly expressed with nuclear localization in the majority of cells compared to normal exocervix ($p<0.001$). The expression of $p16^{INK4A}$ showed a gradual significant increase among pre-neoplastic and neoplastic cervical lesions ($p<0.001$). A positive correlation has been found between the expressions of the $p16^{INK4A}$ and VHR ($p=0.007$).

**Conclusion:** The results suggest that VHR can be considered as a new marker for cancer progression in cervical carcinoma and a potential new target for anticancer therapy. SIL grade was positively related to the expression of $p16^{INK4A}$. Our results confirm that p16 can be a useful biomarker in the diagnosis of cervical neoplasia.

## KEY WORDS:

Squamous cell carcinoma of the cervix, squamous intra-epithelial lesions (SIL) VHR Vaccinia virus VH1-related (VHR) dual-specific protein phosphatase $p16^{INK4A}$ Immunohistochemistry.

## INTRODUCTION:

Cervical cancer is known to develop from premalignant squamous intraepithelial lesions (SIL). It takes nearly 5 to 15 years for only 10–15% of such lesions to progress to invasive cancer (McCance, 2005). In Egypt, available data from different studies provide widely varying estimates on the prevalence of pre-invasive cervical lesions, ranging from 1% (El Mosselhy et al., 1998) to 8% (Younis et al., 1993) with age range from 20-60 years. According to those data

published regarding cancer pathology registry for 2003-2004, invasive cervical lesions represented 1.72% of total malignancies and ranked 11th (Mokhtar, 2007). Many extensive epidemiologic and molecular biologic studies have found that the human papillomavirus (HPV) infection is the most important etiology of cervical cancer. In Egypt, previous small hospital based series confirmed the incorporation of HPV genome in invasive lesions (Ahmed et al., 2001). The persistent infection of high-risk HPV has been implicated in the development of cervical cancer (Lorincz et a., 1992). HPV is known to induce cervical cancer through uncontrolled G1-S transition. The E6 and E7 proteins of high-risk HPV inhibit the p53 and pRb proteins which are cell cycle regulatory proteins controlling G1-S transition (Bosch et al., 1995).

The $p16^{INK4a}$ (p16) is a protein which belongs to the inhibitors of cyclin dependent kinase (CDK) 4 family (INK4a family). By interacting with CDK4 and CDK6, p16 inhibits the formation of cyclin D/CDK4 and 6 complexes. The p16 also functions as a cyclin-dependent kinase inhibitor (CDKI) by inhibiting the CDK-induced phosphorylation of pRb. The phosphorylation of pRb induces the release of a transcription factor E2F from the bound form of E2F and pRb (Nam et al., 2006; Nam et al., 2008). The release of E2F results in G1-S transition. The released E2F stimulates the expression of genes which are involved in G1-S transition. The transition from G1 to S is an extremely important checkpoint in the cell cycle clock because once cells cross this barrier they are committed to progress into S phase (Bartek and Lukas, 2001; Robbins and Cotran, 2004). The inactivation of pRb by E7 causes the p16 overexpression because p16 is regulated by negative feedback of pRb. The $p16^{INK4A}$ gene was found inactivated in a large percentage of tumor cell lines, suggesting that it was indeed a tumor suppressor gene (Kamb et al., 1994; Nobori et al., 1994; Okamoto et al., 1994). $p16^{INK4A}$ overexpression has been demonstrated in cervical cancers. A number of studies have (Tam et al., 1994) demonstrated that $p16^{INK4A}$ may be a useful marker for squamous and glandular epithelial dysplasia in the uterine cervix (Klaes et al., 2001; Dray et al., 2005). Furthermore, expression of $p16^{INK4A}$ appears to correlate with the degree of cervical neoplasia. It was also recently reported that $p16^{INK4A}$ immunostaining can be used for discriminating integrated from non-integrated HPV infections (Agoff et al., 2003; Negri et al., 2004; Dray et al., 2005; Arias-Pulido et al., 2006).

The human genome contains 61 genes for Vaccinia virus H1-like or 'dual-specific' protein phosphatases (DUSPs) (Alnson, 2004). One of these phosphatases is the 185-amino acid residues Vaccinia H1-related (VHR), encoded by the *DUSP3* gene (Ishhibashi et al., 1992), which dephosphorylates and thereby inactivates the mitogen-activated protein kinases (MAPK) Erk and Jnk *in vivo* (Gergnoli et al., 2006; Rahmoni et al., 2006). The levels of VHR fluctuate during the cell cycle. In early G1, VHR is barely detectable then it increases to reach a peak before mitosis. The elimination of VHR by RNA interference resulted in cell cycle arrest in G1/S and G2/M. Such effect of VHR knock-down was counteracted by down modulation of the levels of Erk and Jnk or by modest levels of Mek and Jnk inhibitors. Based on these data, it has been proposed that VHR is important for cell cycle progression as

it tempers Erk and Jnk during the S and G2/M phases of the cell cycle, where excessive activity of these kinases can activate cell cycle check points. Recently, Henkens et al. (2008) have described that the protein level of that newly discovered cell cycle regulator was increased in HSIL and invasive SCC lesions in comparison with normal cervical epithelium.

The current study has been designed to investigate the expression of VHR in a series of pre-neoplastic and neoplastic cervical tissues and to assess its role in the progression of cervical carcinoma. The significance of $p16^{INK4A}$ as a useful diagnostic biomarker was evaluated as well in the same series of cervical tissue samples then the relationship between the two markers was evaluated.

## MATERIALS AND METHOD:

### Patients and tissue specimens

The cases selected for the current study were retrieved from the archives of Pathology laboratory, faculty of Medicine, Minia University Hospital and department of Pathology, faculty of Medicine, Sohag University and represented anonymized, formalin-fixed and paraffin-embedded cervical biopsies diagnosed between the years 2000 and 2006. These cases were selected on the basis of availability of at least one evaluable tissue representative haematoxylin and eosin-stained slide and a paraffin block. For each case, slides were reviewed to confirm the histopathological diagnosis.

The whole series included (12) L-SIL, (18) H-SIL and (20) invasive SCC that have been obtained from hysterectomy or conization specimens (obtained by large loop excision of the transformation zone). Cases of invasive SCC were examined by two independent pathologists, classified and graded according to the World Health Organization (WHO) criteria. Tissue blocks containing only small or otherwise inadequate samples were excluded from the study. Normal exocervix (10) specimens were obtained from cases of hysterectomy performed for non-cervical pathologies.

### Positive and negative controls

One positive control tissue section for each antibody was processed in the same manner as the patient tissue samples and was included in each staining run. Squamous cell carcinoma was used as a positive control for both VHR and p16 antibodies.

The negative control was used to verify the specificity of the primary antibody where a squamous cell carcinoma section was processed through the procedure of immunostaining but the primary antibody was omitted and phosphate buffer solution was added instead. Absence of specific staining in the negative control sections confirms lack of secondary antibody cross-reactivity with other non-target cellular components.

### Immunohistochemistry

Four μm-thick serial sections from archival paraffin-embedded tissues were freshly cut, placed on poly-L-lysine coated slides, de-paraffinized with xylene, and rehydrated through graded alcohol. Endogenous peroxidase activity was blocked by incubation with 0.3% Hydrogen peroxide/Methanol for 30 minutes. Antigen retrieval was achieved by microwave treatment, where the slides were placed in sodium citrate buffer (0.01M, pH 6.0) for 10 minutes. The appropriate antibody was diluted at its optimum concentration in PBS containing 5% (w/v) bovine serum

albumin. Tissue sections were then incubated with antibodies against VHR (Clone 237020 dilution 1:2500, R&D Systems, Minneapolis, MN; the antibody was generously provided by Dr. M Smith) and p16 (mouse monoclonal antibody, Ready to use, Labvision) for 2 hours at room temperature. Thereafter, the slides were thoroughly rinsed with PBS and a biotinylated goat anti-mouse secondary antibody was applied to sections for 30 minutes at room temperature. Visualization of the reaction was performed with an avidin-biotin complex immunoperoxidase system using 3, 3' diamino-benzidine as a chromogen for 10 minutes. Then, the slides were rinsed in running tap water and lightly counterstained with Mayer's haematoxylin. Finally, sections were dehydrated in graded alcohol, cleared in xylene and mounted with DPX.

**Scoring of immunohistochemical staining**

**Scoring of VHR immunostaining**

The VHR immunostaining was scored semi-quantitatively as previously demonstrated by Henkens et al. (2008). For staining intensity, 0 represented samples in which no staining was detectable, whereas 1+, 2+, and 3+ denoted samples with low, moderate, and strong nuclear and/or cytoplasmic staining, respectively. For staining extent, in normal exocervix and the various grades of SIL, 1+ represented VHR expression that was detectable in the lower 1/3 of the epithelium, whereas 2+ denoted detectable VHR expression in the lower 2/3 of the epithelium and 3+ represented detectable immunoreactivity that reached the upper 1/3 of the epithelial layer. For the extent of staining in SCC, 1+ represented samples in which VHR expression was detectable in up to 33% of the cells whereas 2+ denoted samples in which > 33–66% of the cells showed detectable VHR expression and 3+ represented those in which more than 66% of the cells were stained. In order to provide a global score for each case, the results obtained with the two scales were multiplied, yielding a single scale with steps of 0 to 9. The immunostaining was evaluated by two independent observers (MIA and EMS) and discrepancies were resolved by consensus.

**Scoring of p16 immunostaining**

Interpretation of the staining results was done by evaluation of the percentage of p16 positive cells (combination of nuclear and cytoplasmic staining) in the highest expression area (hot spot) then grading was performed as described by Conceicao et al. (2006) as follows: grade 0= no cells were stained; grade 1= < 50% of cells were positive, grade 2= 50-80% of cells exhibited expression, and grade 3= > 80% of cells**.**

**RESULTS:**

**VHR expression**

The mean age for each group included in this study was (35.7) years for LSIL, (41) years for HSIL and (49) years for SCC. VHR expression at the protein level was studied using immunohistochemistry technique in a panel of anonymous, formalin-fixed and paraffin-embedded tissues of normal exocervix (*n* =10), low-grade SILs (*n* =12), high-grade SILs (*n* =18) and invasive squamous cell carcinoma SCCs (*n* =20). Semiquantitative analysis of VHR immunostaining was employed. The VHR was expressed at very low level only in the cytoplasm of para-basal cell layers of the normal squamous epithelium (Figure 1). On the other hand, both nuclear and cytoplasmic staining was observed in LSIL and HSIL (Figures 2 & 3, respectively). The VHR was signify-

cantly up-regulated in squamous intraepithelial lesions (SILs) of the cervix in low grade SIL L-SIL ($p<0.05$) and high grade SIL (H-SIL) ($p<0.0001$) in comparison to normal exocervix. In cases of invasive squamous cell carcinoma, VHR was highly expressed with both cytoplasmic as well as nuclear localization in the malignant cells (Figure 4) and such expression was statistically significant ($p <0.0001$) when compared with its expression in normal exocervix (Table 1).

**Table (1):** Immunohistochemical analysis of VHR expression in normal, LSIL, HSIL and SCC cervical tissue samples by using a semi-quantitative scoring method for evaluation

| **Diagnosis** | **VHR immunostaining score*** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| **Normal (n=10)** | - | 3 | 4 | 2 | 1 | - | - | - | - | - |
| **Low-SIL (n=12)** | - | - | 6 | - | 3 | 2 | 1 | - | - | - |
| | *$P^{**} < 0.05$* | | | | | | | | | |
| **High-SIL (n=18)** | - | - | 1 | 3 | 2 | 3 | 6 | 2 | - | - |
| | *$P^{**} < 0.0001$* | | | | | | | | | |
| **SCC (n=20)** | - | - | 1 | 2 | 4 | 3 | 5 | 5 | - | - |
| | *$P^{**} < 0.0001$* | | | | | | | | | |

* Samples were assigned an immunohistochemical score (0-9) according to the results obtained with a multiplied two scales (staining intensity x staining extent). For staining intensity, 0 = no staining was detectable, 1+= low, 2+=moderate, and 3+=strong cytoplasmic and/or nuclear staining was detected. For staining extent, in normal exocervix and the various grades of SIL, 1+ = VHR expression was detectable in the lower 1/3 of the epithelium, 2+= detectable VHR expression in the lower 2/3 of the epithelium and 3+= represented detectable immunoreactivity that reached the upper 1/3 of the epithelial layer. For the extent of staining in SCC, 1+= VHR expression was detectable in up to 33% of the cells, 2+= > 33–66% of the cells showed detectable expression and 3+= > 66% of the cells were stained.

** P value was obtained by correlation of the immunohistochemical score for each group individually (LSIL, HSIL and SCC) with normal cervical epithelium

**$p16^{INK4}$ expression**

The expression of $p16^{INK4A}$ in cervical epithelium was characterized by variable, weak to strong, diffuse cytoplasmic and nuclear staining. There was no difference in the staining intensity among different epithelial layers. Clear and distinctive positive immuno-staining was observed only in dysplastic and neoplastic cells. Both normal squamous epithelial cells of the exocervix and stromal cells were consistently negative.

The expression of $p16^{INK4A}$ was positively associated with SIL grade, as the SIL grade was higher, stronger expressions was observed ($p < 0.001$). On the other hand, it was totally absent (100%) in normal

cervical epithelium. In addition, there was positive correlation between the expression of the $p16^{INK4A}$ and VHR (p=0.007).

**Correlation between histological Diagnosis and % of cells positive for P16**

**A. Normal epithelia of cervical mucosa**

P16 showed negative expression in all ten cases (100%) of normal epithelia of cervical mucosa (Figure 5-A).

**B. Low SIL**

P16 expression in LSIL in comparison with that of normal squamous cervical epithelium is displayed in Table (2). P16 expression in total number of LSIL showed positive expression (Figure 5-B) in 8/12 cases (66.7%), these positive cases were categorized into three groups according to their scores, two cases (16.6%) had score1, three cases (25%) got score 2 and three cases (25%) had score 3. The remaining 4 cases (33.3%) showed negative expression. The distribution of staining in all LSIL cases, showed at least focal $p16^{INK4a}$ immunoreactivity. $p16^{INK4a}$ immunoexpression were confined to the lower half of the epithelium in most of the cases. These results revealed a gradual significant increase in the expression of p16 in LSIL cases, as compared to expression of p16 in normal epithelia of cervical mucosa (P<0.01), (From 100% negativity in normal epithelia of cervical mucosa to 66.7% positivity in LSIL).

**Table (2):** P16 expression in LSIL in comparison with that of normal squamous cervical epithelium

| Diagnosis | Percentage of positive cells for P16 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Grade 0 | | Grade 1 | | Grade 2 | | Grade 3 | |
| | n | % | n | % | n | % | n | % |
| **Normal (n=10)** | 10 | 100% | 0 | | 0 | | 0 | |
| **Low SIL( n=12)** | 4 | 33.3% | 2 | 16.6% | 3 | 25% | 3 | 25% |
| **P value <0.05** | <0.01* S | | | | | | | |

Grades: 0, none of cells showed expression; 1, less than 50 % of cells were stained; 2, 50-80% % of cells were positive; 3 more than 80 % of positive cells were detected.
***** S= significant

**C. High SIL**

P16 expression in total number of HSIL showed positive expression (Figure 6) in 18/18 (100%) cases (Table 3). The positive cases were categorized into three groups according to their scores, two cases (11.1 %) with score 1, two cases (11.1%) with score 2 and 14 cases (77.8%) with score 3. The distribution of staining showed at least diffuse, strong staining for $pl6^{INK4a}$ in the lower two thirds of the epithelium up to diffuse full thickness pattern. These results show a gradual increase in the frequency as well as the intensity of expression of p16 in HSIL cases, as compared to expression of p16 in LSIL cases (P<0.01).

**Table (3):** P16 expression in HSIL in comparison with that detected in LSIL cases

| Diagnosis | Percentage of positive cells for P16 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Grade 0 | | Grade 1 | | Grade 2 | | Grade 3 | |
| | n | % | n | % | n | % | n | % |
| Low SIL (n=12) | 4 | 33.3% | 2 | 16.6% | 3 | 25% | 3 | 25% |
| HSIL (n=18) | 0 | | 2 | 11.1% | 2 | 11.1% | 14 | 77.8% |
| P value | <0.01* S | | | | | | | |

Grades: 0, none of cells showed expression; 1, less than 50 % of cells were stained; 2, 50-80% % of cells were positive; 3, more than 80 % of positive cells were detected.
*S= Significant

**D. Squamous cell carcinomas**

P16 expression in total number of SCC showed positive expression (Figure 7) in 19 (95%) out of 20 cases, while only one case (5%) showed negative expression (Table 3). The positive cases were categorized into three groups according to their scores, 2 cases (10%) with score 1, seven cases (35%) with score 2 and 10 cases (50%) with score 3. There is apparent gradual increase in the expression of p16 in SCC cases as compared to expression of p16 in LSIL cases ($P<0.05$) (From 33.3% negativity in LSIL cases to only 5% negativity in SCC cases).

**Table (4):** P16 expression in SCC cases in comparison with that detected in LSIL

| Diagnosis | Percentage of positive cells for P16 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Grade 0 | | Grade 1 | | Grade 2 | | Grade 3 | |
| | n | % | n | % | n | % | n | % |
| Low SIL (n=12) | 4 | 33.3% | 2 | 16.6% | 3 | 25% | 3 | 25% |
| SCC (n=20) | 1 | 5% | 2 | 10% | 7 | 35% | 10 | 50% |
| P value | 0.002* | | | | | | | |

Grades: 0, none of cells showed expression; 1, less than 50 % of cells were stained; 2, 50-80% % of cells were positive; 3, more than 80 % of positive cells were detected.
* Significant

**E. Correlation between all histo-pathological variants and percentage of cells positive for P 16 staining**

Concerning different histopathological variants (Table 4), it was shown that the positive expression for p16 in pre-neoplastic and neo-plastic histopathological cervical lesions was in gradual significant increase among different histo-pathological variants (P < 0.001). In the normal epithelia of cervical mucosa, there was complete lack of expression in all cases (i.e. negative expression in 100%). In pre-neoplastic and neoplastic tissues, p16 positive immunoreactivity could be detected in 10/12 (83.3%) of LSIL cases, in all 18 cases (100%) of HSIL and in 19/20 of SCC cases (95%). Also there was gradual increase in the positive cases with scores 2 and 3 from two cases(16.6%) in LSIL cases, then it reached maximum positivity rate where it was expressed in16 cases (88.1%) of HSIL cases and 16 cases (80%) of SCC cases. This denotes that the scoring of p16 is increased with the progression from normal to LSIL and from LSIL to HSIL and SCC.

**Table (5):** Correlation between the grades of p16 expression among different groups involved in the study

| Diagnosis | Percentage of positive cells for P16 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Grade 0 | | Grade 1 | | Grade 2 | | Grade 3 | |
| | n | % | n | % | n | % | n | % |
| **Low SIL (n=12)** | 10 | 83.3% | 0 | | 1 | 8.3% | 1 | 8.3% |
| **HSIL (n=18)** | 0 | | 2 | 11.1% | 2 | 11.1% | 14 | 77.8% |
| **SCC (n=20)** | 1 | 5% | 2 | 10% | 7 | 35% | 10 | 50% |
| **P value** | <0.001* | | | | | | | |

Grades: 0, none of cells showed expression; 1, less than 50 % of cells were stained; 2, 50-80% % of cells were positive; 3, more than 80 % of positive cells were detected.
* Significant

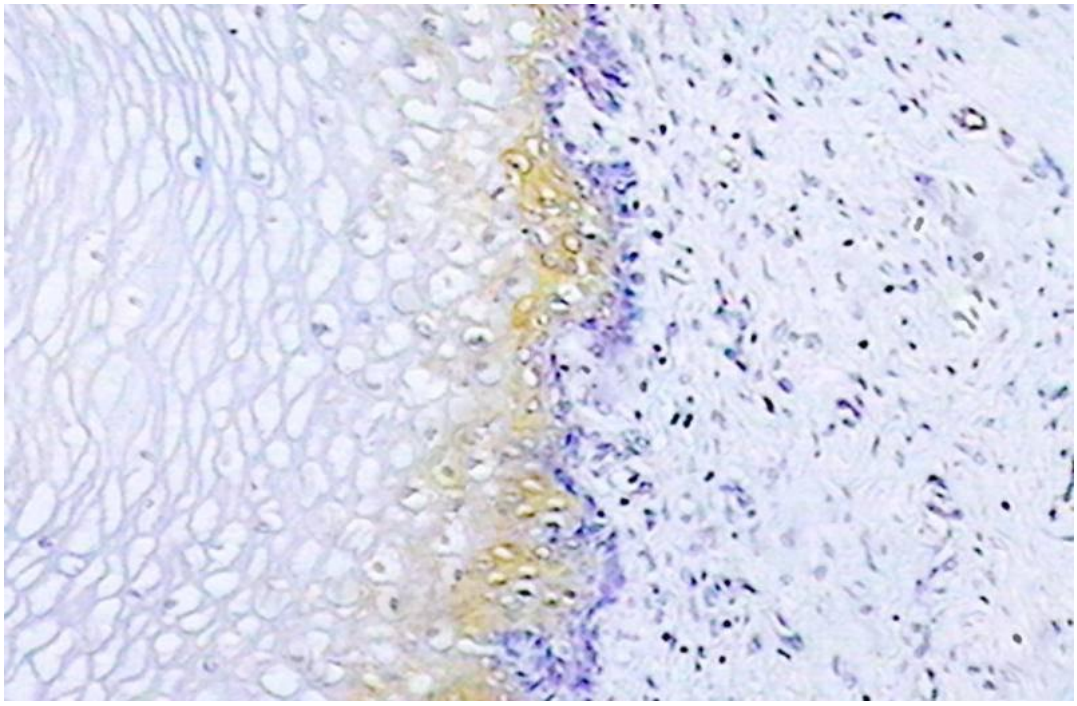

**Figure (1) Faint expression of VHR in normal cervical epithelia**

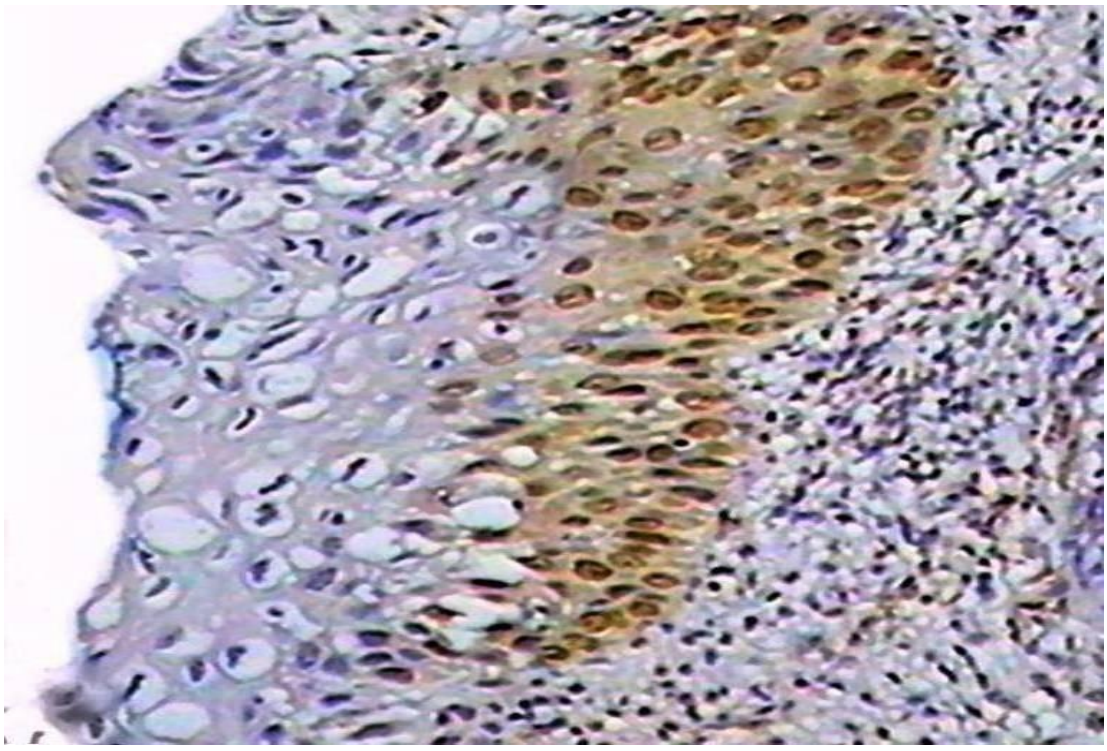

**Figure (2) Positive immunostaining of VHR in LSIL**

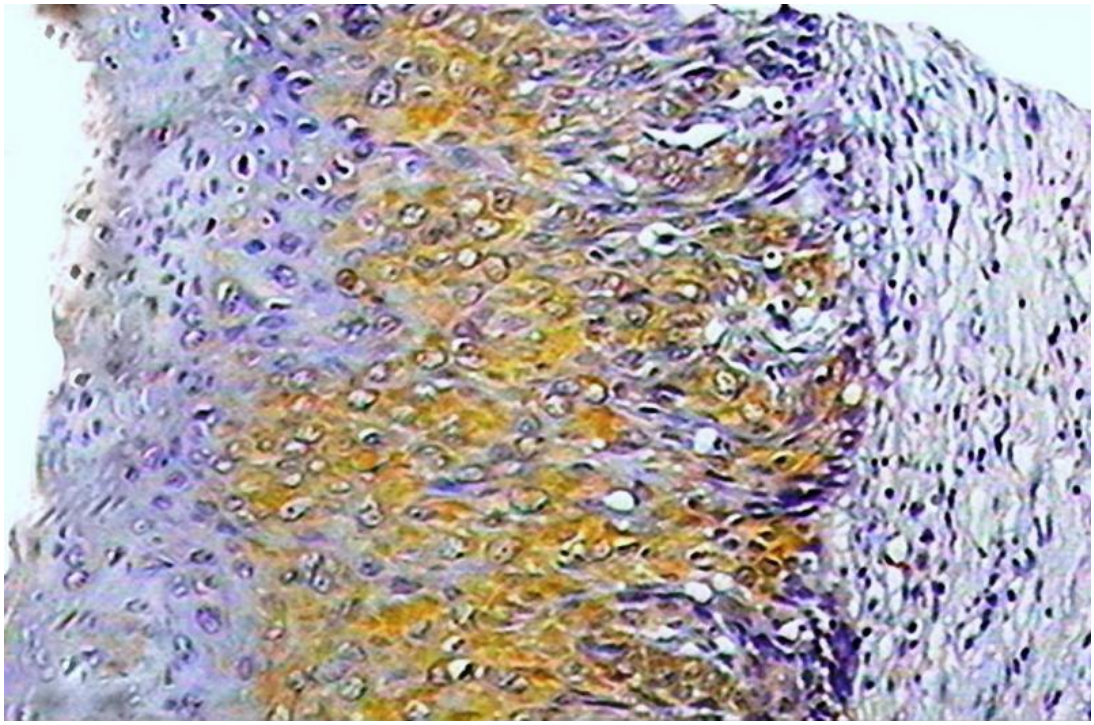

**Figure (3) Strong staining of VHR in HSIL**

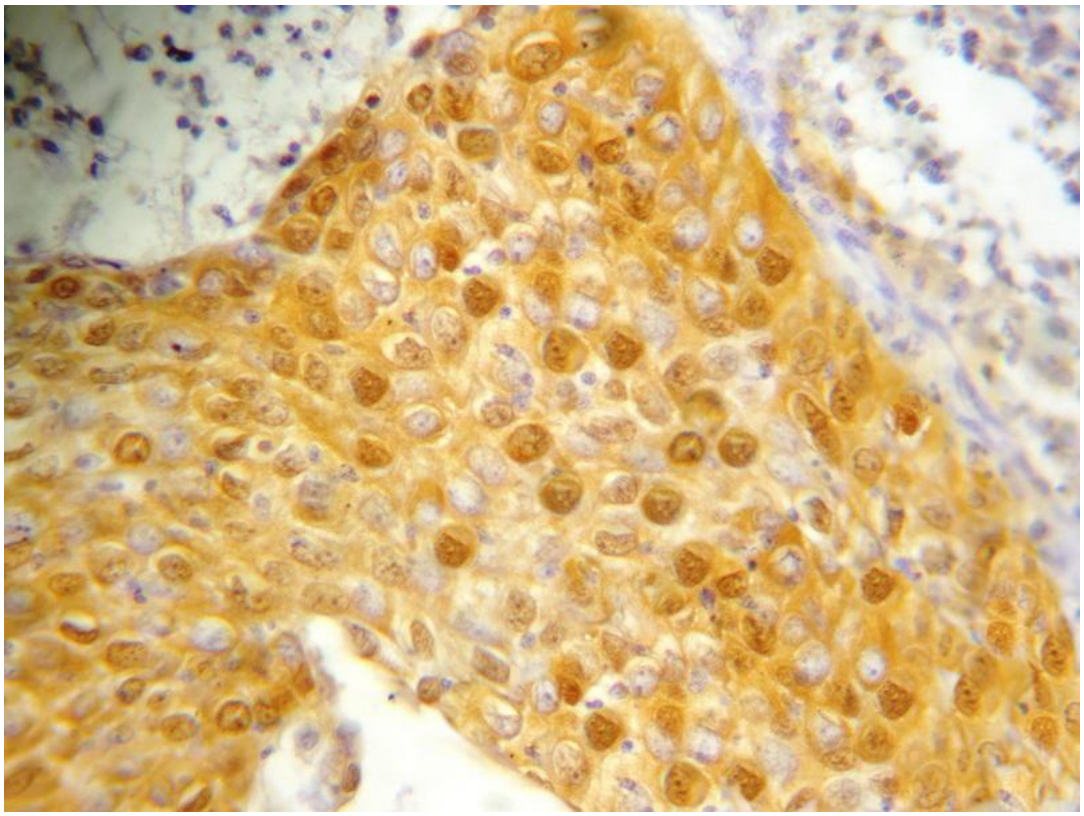

**Figure (4) Immuoreactivity of VHR in invasive SCC**

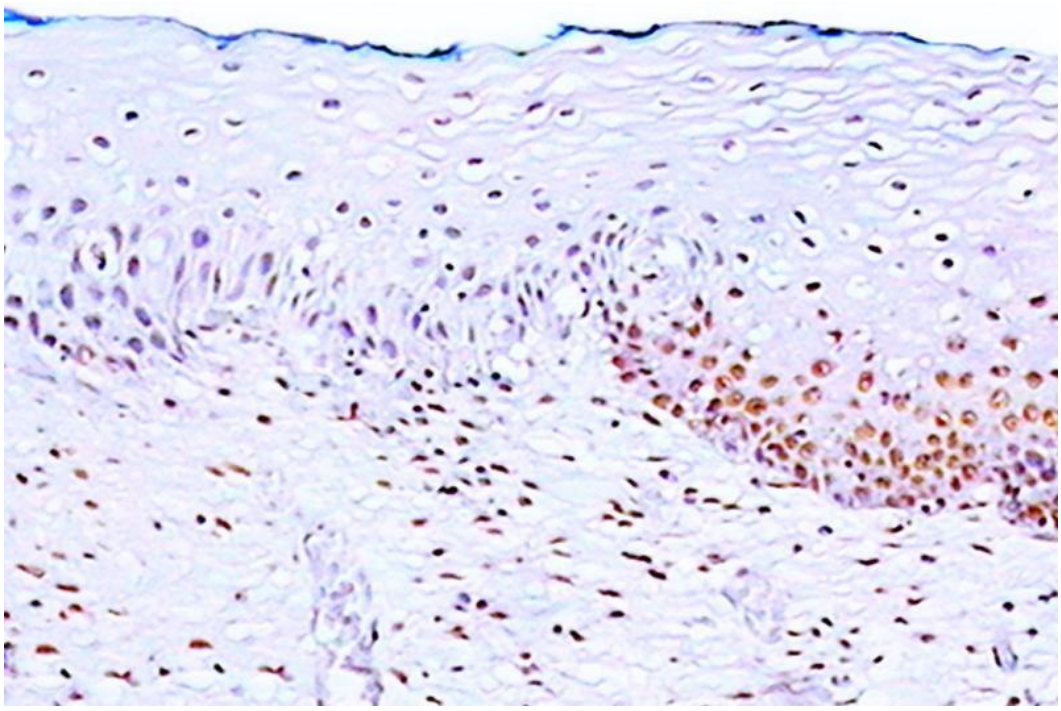

**Figure (5) Complete absence of p16 expression in normal epithelium (left) while adjacent area of LSIL shows positive immunoreactivity (right)**

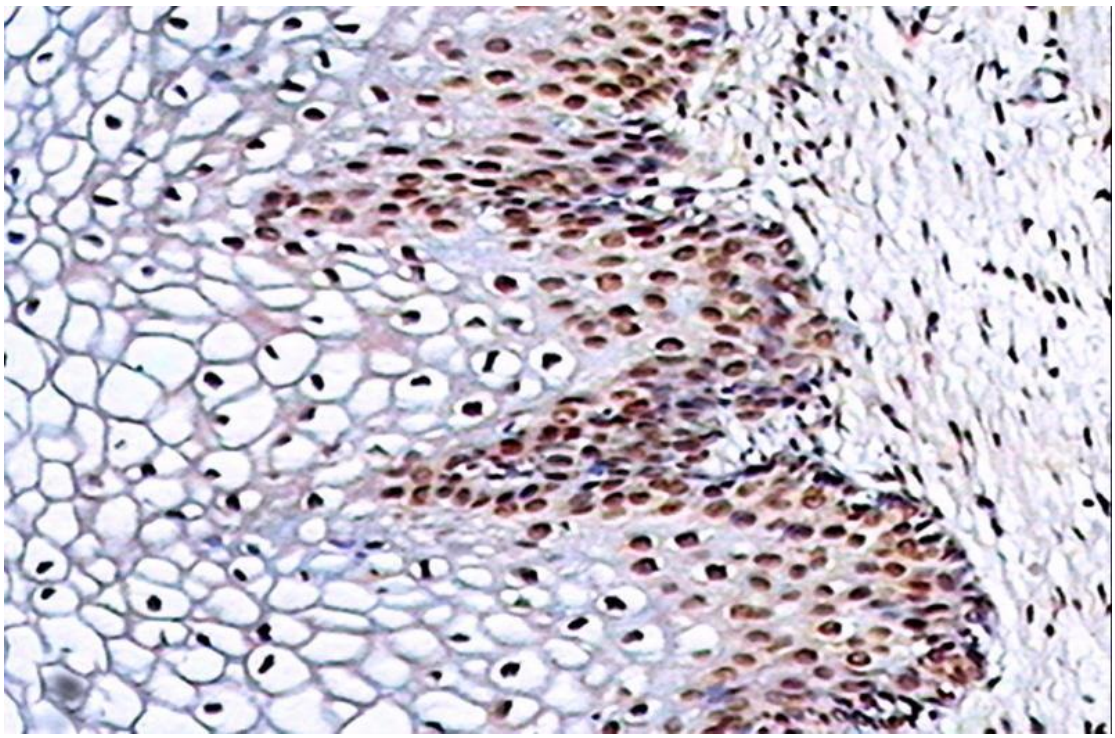

**Figure (5-B) Higher magnification of LSIL positive immunostaining in previous case in Figure (5-A)**

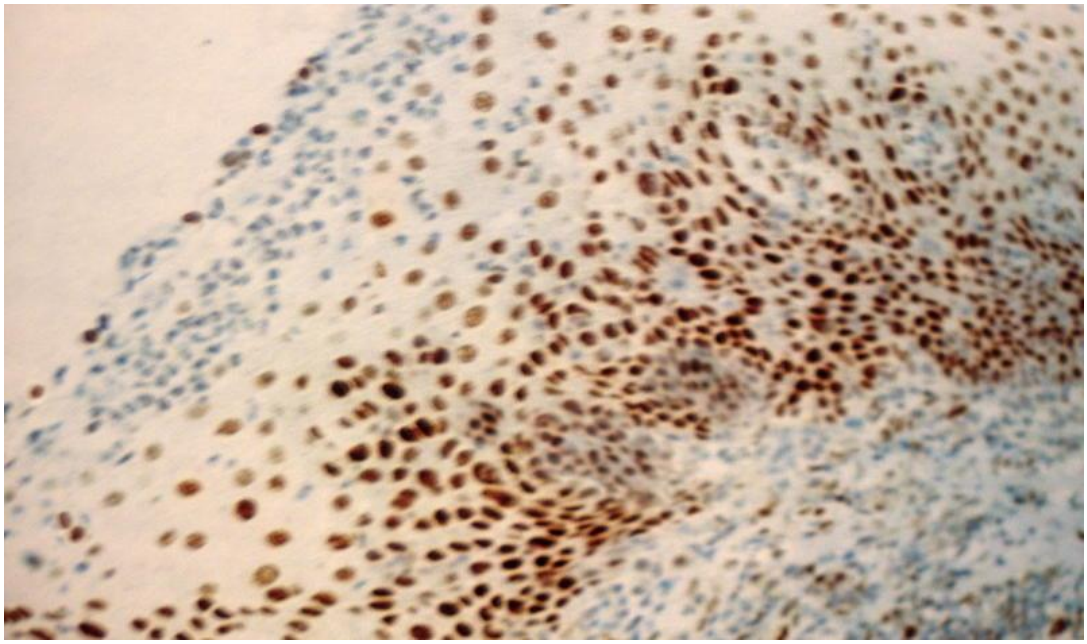

**Figure (6) Strong nuclear staining of p16 in HSIL**

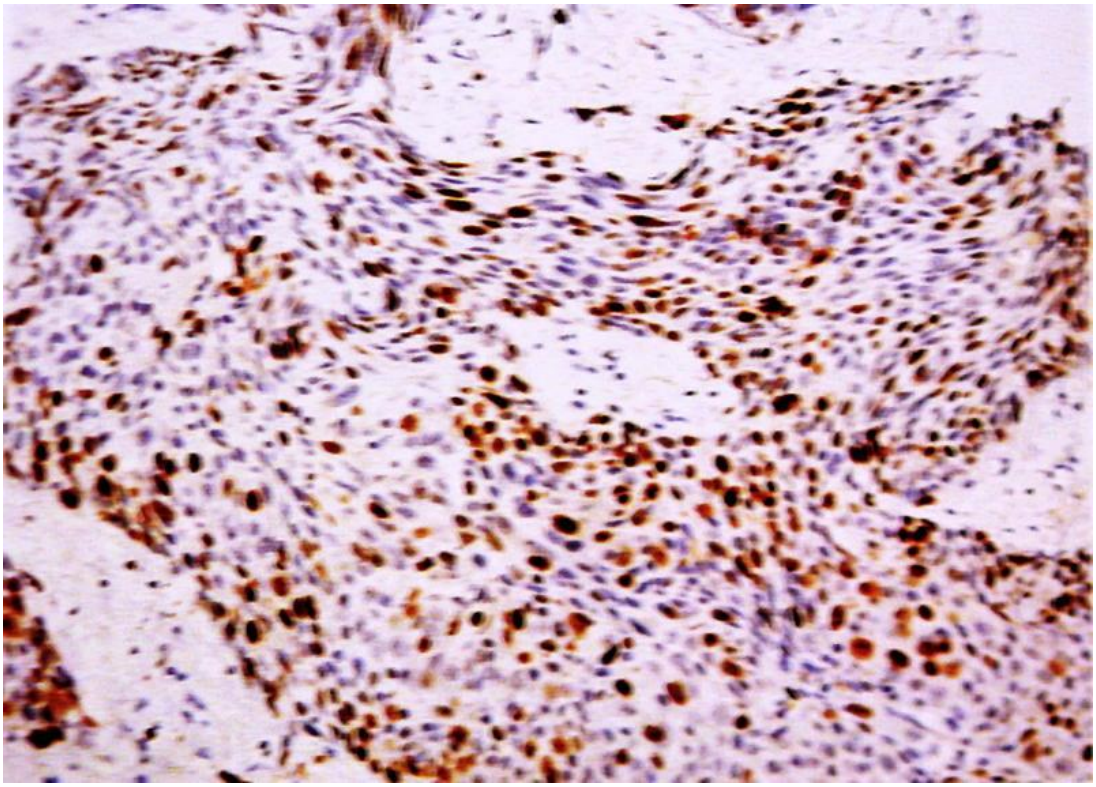

**Figure (7) Immunoexpression of p16 in invasive SCC**

## DISCUSSION:

The development and progression of cervical carcinoma is dependent on both genetic and epigenetic events, including alterations in the cell cycle machinery at various checkpoints. It has been reported that cervical carcinoma is associated with aberrant regulation of cyclins D1 and E, p16, p21 and p27 (Milde-langosch et al., 2001). Recently, the newly described cell cycle regulator Vaccinia H1-related (VHR) protein, encoded by the *DUSP3* gene (Ishibashi et al., 1992), has been reported to be important for cell cycle progression as it tempers Erk and Jnk during the S and G2/M phases of the cell cycle, where excessive activity of these kinases can activate cell cycle check points (Cerignoli et al., 2006; Rahmouni et al., 2006). In the current study we have investigated the expression of VHR in a series of pre-neoplastic intraepithelial squamous lesions (LSIL and HSIL) and invasive squamous cell carcinoma (ISCC) in order to assess its role in progression of cervical carcinoma. The significance of $p16^{INK4A}$ as a useful diagnostic biomarker was evaluated as well in the same series of cervical tissue samples then the relationship between the two markers was evaluated.

In this study, VHR expression at the protein level has been investigated using immunohistochemistry technique in a series of formalin-fixed and paraffin-embedded tissues of normal exocervix (*n* =10), low-grade SILs (*n* =12), high-grade SILs (*n* =18) and invasive squamous cell carcinoma SCCs (*n* =20). The VHR was expressed at very low level only in the cytoplasm of para-basal cell layers of the normal squamous epithelium while both nuclear and cytoplasmic staining was observed in LSIL and HSIL. The VHR was significantly up-regulated in squamous intraepithelial lesions (SILs) of the cervix in low grade SIL L-SIL ($p<0.05$) and high grade SIL (H-SIL) ($p<0.0001$) in comparison to normal exocervix. In cases of invasive squamous cell carcinoma, VHR was highly expressed with predominant nuclear localization in the malignant cells and such expression was statistically significant ($p <0.0001$) when compared with its expression in normal exocervix. These results are in agreement with those reported by Henkens et al (2008) who found that in the primary cervical carcinomas, VHR was highly expressed in many epithelial cells of all the H-SIL analyzed in that study as well as in SCCs, where the intensely stained cells increased markedly in SCC cases. Taken together such results suggest that VHR can be considered as a new marker for cancer progression in cervical carcinoma.

The authors mentioned as well that the staining for VHR was compared to that of p16, in spite of not showing such data, and they found that all the VHR-positive cells were also positive for p16 which is considered as a marker of cervical (pre)cancer cells (Aguilar-Lemarroy et., 2002). Such finding was explained on the basis of that VHR is barely detectable in G1 phase cells, but gradually increases during the prog-ression of the cells to G2/M phase (Rahmouni et al. (2006). Thus, they proposed that similar like p16, VHR can be considered as a marker of cells in cycle (S, G2 and M phases). Interestingly, in the present study, we have found a positive correlation between the expression of the $p16^{INK4A}$ and VHR ($p=0.007$).

In our study we have looked at the association between $p16^{INK4a}$ expression and cervical neoplasia. Immunohistochemical expression of $p16^{INK4a}$ was detected only in dysplastic/neoplastic cells, and was never observed in normal cervical epithelium. In pre-neoplastic and

neoplastic lesions, it was shown that the positive expression for p16 is in gradual significant increase among different histopathological variants ($P < 0.001$), In the normal epithelia of cervical mucosa, there is complete negative expression in all cases (100%) i.e. no positive expression, in LSIL cases , it showed positive expression 10/12 (83.3%) of LSIL cases, in all 18 cases (100%) of HSIL and in 19/20 of SCC cases (95%). Also there was gradual increase in the positive cases with scores 2 and 3 from two cases (16.6%) in LSIL cases, then it reached maximum positivity rate where it was expressed in16 cases (88.1%) of HSIL cases and 16 cases (80%) of SCC cases. These results are similar to those found in one study where p16 was found in eight out of 13 cases of LSIL (61%), in HSIL cases, it showed positive expression in nine out of ten cases (90%) and in SCC cases, it showed positive expression in 19 out of 20 cases(95%). Also there was gradual increase in the positive cases that with score 2, 3 from seven cases (53.83%) in LSIL cases, to seven cases (70%) in HSIL cases and reached maximum positivity rate, 16 case(80%) in SCC cases. This denotes that the scoring of p16 is increased with the progression from normal to LSIL and from LSIL to HSIL, and SCC. Thus, $p16^{INK4a}$ expression appears to be a robust, specific and sensitive biomarker of cervical neoplasia, confirming the results of previous studies (Klaes et al., 2001; Dray et al., 2005; Agoff et al., 2003 and Negri et al., 2004). Although other pathways cannot be ruled out, increased expression of $p16^{INK4a}$ in the setting of CIN probably occurs mainly as a result of inactivation of RB by high-risk HPVs. Circumstantial support for this premise comes from the observation that increasingly high $p16^{INK4a}$ expression scores were seen in cervical specimens showing higher grades of CIN or invasive carcinoma, lesions known to be closely associated with high-risk HPV infection.

Henkens et al (2008) has demonstrated that VHR is a cytosolic protein in primary keratinocytes, but localizes both in the cytoplasm and nucleus in cervix cancer cells. VHR does not contain recognizable nuclear localization or nuclear export sequences, but is small enough to passively diffuse into the nucleus. Thus, its exclusion from the nucleus in primary keratinocytes likely is an active process. The authors declared that preliminary results have shown that VHR associates with cyclins (unpublished observation), perhaps explaining its retention in the nuclear or cytosolic compartments in normal versus malignant cells. Thus, as previously proposed, it appears that VHR location may be connected to cell cycle-dependent transport of cyclins or dysregulation of cyclins in cancer (Milde-Langosch et al., 2001)

It has been reported that the HPV negative cell line C33A and HPV negative adenocarcinomas are p16INK4A positive, which indicates that a non- HPV dependent p16INK4A expression pathway may also exist (Kales et al., 2001). Interestingly, VHR was increased at the protein level in five different cervix cancer cell lines positive (HeLa, CaSki and SiHa) or negative (C33 and HT3) for HPV compared to primary keratinocytes prepared from hysterectomies (Hekens et al (2008). Such results have shown that VHR expression is not related to the high risk human papilloma virus.

Rahmouni et al. (2006) and Cerignoli et al. (2006) have reported that the human Vaccinia H1-related (VHR) dual-specific protein tyrosine

phosphatase regulates cell-cycle progression and is itself modulated during the cell cycle. Using RNA interference (RNAi), they demonstrated that cells lacking VHR arrest at the G1-S and G2-M transitions of the cell cycle and show the initial signs of senescence, such as flattening, spreading, appearance of autophagosomes, beta-galactosidase staining and decreased telomerase activity. In agreement with this notion, cells lacking VHR were found to upregulate p21 (Cip-Waf1), whereas they downregulated the expression of genes for cell-cycle regulators, DNA replication, transcription and mRNA processing. Loss of VHR also caused a several-fold increase in serum-induced activation of its substrates, the mitogen-activated protein (MAP) kinases Jnk and Erk. VHR-induced cell-cycle arrest was dependent on this hyperactivation of Jnk and Erk, and was reversed by Jnk and Erk inhibition or knock-down. They concluded that VHR is required for cell-cycle progression as it modulates MAP kinase activation in a cell-cycle phase-dependent manner. Thus, by elevating the levels of VHR, cancer cells would prevent Erk and Jnk from becoming too active in S through G2, while still allowing them to drive G1 progression. In support of this view, it was recently reported that both Erk and Jnk are less active in invasive cervical malignant cells than in premalignant epithelial lesions (Engelbrecht et al., 2006)

VHR is regulated during cell cycle progression and because its suppression by RNA interference (Rahmouni et al., 2006) halts cellular proliferation and induces cellular senescence, and, more importantly, because of its overexpression in the cervical cancer, it has been suggested that VHR may be a good target for anticancer therapy. It has been proposed that cervical cancer cells that have adapted to high levels of VHR expression can be sensitive to VHR small inhibitors and that untransformed cells are much less dependent on VHR for proliferation and survival (Henken et al., 2008). The investigators mentioned that they have already developed novel multidentate small molecule inhibitors of VHR that inhibit its enzymatic activity at nanomolar concentrations *in vitro*, and are active at low micromolar concentrations on several cell lines and primary cells. In that unpublished work they have tested these inhibitors on HeLa and primary keratinocytes and demonstrated that they halt HeLa cells proliferation while they have no effect on primary keratinocytes.

VHR has been investigated in other tissues like breast and prostate to elucidate its proposed role. Recently, Arnoldussen et al. (2008) systematically investigated the possible regulation of mitogen-activated protein kinase phosphatases/dual-specificity phosphatases during apoptosis of LNCaP cells and found that Vaccinia H1-related protein (VHR/DUSP3) is up-regulated by androgens during inhibition of apoptosis in androgen-dependent LNCaP cells, but not in androgen-independent DU145 cells. They showed as well that VHR is increased in prostate cancer tissues compared with normal prostate. All together their data suggest that VHR may have a role in prostate cancer progression. In breast cancer cells, Hao and El-Shamy (2007) have shown that, overexpression of BRCA1-IRIS induces Cyclin D1 overexpression and increases cell proliferation. BRCA1-IRIS overexpression decreases the expression of the dual specificity phosphatase, DUSP3/VHR, an endogenous inhibitor of several MAPKs, including c-Jun N-terminal kinase.

Although, the mechanism by which BRCA1-IRIS overexpression accomplishes that is not yet known, it is sufficient to induce Cyclin D1 overexpression in a human mammary epithelial cell model. Cyclin D1 overexpression could be blocked by co-overexpression of VHR in those cells.

## CONCLUSION:

The results of the current study support the findings of other reports that VHR is an important cell cycle regulator. Loss of this phosphatase causes senescence and prolonged hyperactivation of Erk and Jnk pathways. The results of this study together with published recent reports suggest that VHR can be considered as a new marker for cancer progression in cervical carcinoma and a potential new target for anticancer therapy by induction of senescence in tumour cells. SIL grade was positively correlated to the expression of $p16^{INK4A}$. Our results confirm that p16 can be a useful biomarker in the diagnosis of cervical neoplasia.

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## REFERENCES:

1. Aguilar-Lemarroy A, Gariglio P, Whitaker NJ, Eichhorst ST, zur Hausen H, Krammer PH, Rösl F: Restoration of p53 expression sensitizes human papillomavirus type 16 immortalized human keratinocytes to CD95-mediated apoptosis. *Onco-gene* 2002; 21(2):165-75.
2. Ahmed MI, Salahy EE, Fayed ST, El-Hefnawy NG, Khalifa A: Human papillomavirus infection among Egyptian females with cervical carcinoma: relationship to spontaneous apoptosis and TNF-alpha. Clin Biochem 2001, 34:491-498.
3. Alonso A, Sasin J, Bottini N, Friedberg I, Osterman A, Godzik A,Hunter T, Dixon J, Mustelin T: Protein tyrosine phosphatases in the human genome. *Cell* 2004, 117(6):699-711.
4. Arias-Pulido H, Peyton CL, Joste NE, Vargas H, Wheeler CM: Human papillomavirus type 16 integration in cervical carcinoma in situ and in invasive cervical cancer. *J Clin Microbiol* 2006, 44(5):1755-1762.
5. Arnoldussen YJ, Lorenzo PI, Pretorius ME, Waehre H, Risberg B, Maelandsmo GM, Danielsen HE, Saatcioglu F. The mitogen-activated protein kinase phosphatase vaccinia H1-related protein inhibits apoptosis in prostate cancer cells and is overexpressed in prostate cancer. Cancer Res. 2008; Nov 15;68(22):9255-64.
6. Bartek J and Lukas J (2001): Mammalian G1- and S-phase checkpoints in response to DNA damage. Curr Opin Cell Biol; 13:738-750.
7. Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, et al. Prevalence of human papillomavirus in cervical cancer: A worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. J Natl Cancer Inst 1995; 87: 796-802.
8. Cerignoli F, Rahmouni S, Ronai Z, Mustelin T. Regulation of MAP kinases by the VHR dual-specific phosphatase: implications for cell growth and differentiation. Cell Cycle. 2006; 5(19):2210-5.
9. Dray M, Russell P, Dalrymple C, Wallman N, Angus G, Leong A,Carter J, Cheerala B: p16(INK4a) as a complementary

marker of high-grade intraepithelial lesions of the uterine cervix. Experience with squamous lesions in 189 consecutive cervical biopsies. *Pathology* 2005, 37(2):112-124.
10. Dyson N, Howley PM, Munger K, Harlow E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 1989; 243: 934-7.
11. El Mosselhy MH, Moselhy MM, Amin A, Ouda-Pasha E, Abdel Hamid, Hussein MH: Prevelance of reproductive tract infections among married women of reproductive age. National Population Council/El Galaa Teaching Hospital, Egypt; 1998.
12. Engelbrecht AM, Gebhardt S, Louw L: Ex vivo study of MAPK profiles correlated with parameters of apoptosis during cervical carcinogenesis. *Cancer Lett;* 2006, 235(1):93-9.
13. Hao L and ElShamy WM. BRCA1-IRIS activates cyclin D1 expression in breast cancer cells by downregulating the JNK phosphatase DUSP3/VHR. Int J Cancer. 2007 Jul 1; 121(1):39-46.
14. Henkens R, Delvenne P, Arafa M, Moutschen M, Zeddou M, TautzL, Boniver J, Mustelin T and Rahmouni S. Cervix carcinoma is associated with an up-regulation and nuclear localization of the dual-specificity protein phosphatase VHR. BMC Cancer 2008, 8:147
15. Ishibashi T, Bottaro DP, Chan A, Miki T, Aaronson SA: Expression cloning of a human dual-specificity phosphatase. *Proc Natl Acad Sci USA* 1992, 89(24):12170-4.
16. Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U, Dallenbach-Hellweg G, Schmidt D, von Knebel Doeberitz M: Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. *Int J Cancer* 2001, 92(2):276-284.
17. Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS 3rd, Johnson BE, Skolnick MH: A cell cycle regulator potentially involved in genesis of many tumor types. *Science* 1994, 264(5157):436-440.
18. Lorincz AT, Reid R, Jenson AB, Greenberg MD, Lancaster W, Kurman RJ. Human papillomavirus infection of the cervix: Relative risk associations of 15 common anogenital types. Obstet Gynecol 1992; 79: 328-37.
19. Milde-Langosch K, Riethdorf S, Kraus-Pöppinghaus A, Riethdorf L,Löning T: Expression of cyclin-dependent kinase inhibitorsp16MTS1, p21WAF1, and p27KIP1 in HPV-positive and HPV-negative cervical adenocarcinomas. *Virchows Arch* 2001, 439(1):55-61.
20. McCance DJ: Human papillomaviruses and cell signaling. *Sci STKE* 2005, 288:pe29.
21. Mokhtar N, Gouda I and Adel I. Cancer Pathology Registry '2003-2004' And Time Trend Analysis. Department of Pathology, NCI, Cairo University. 2007; P 19, 21
22. Nam EJ, Kim HY, Kim SW, Yoon BS, Kim JH, Kim YT, et al. Relationship between p16INK4a, pRb and high risk PV infection and recurrence. Korean J Obstet Gynecol 2006; 9:1437-45.
23. Negri G, Vittadello F, Romano F, Kasal A, Rivasi F, Girlando S, Mian C, Egarter-Vigl E: p16INK4a expression and progression risk of low-grade intraepithelial neoplasia of the cervix uteri. *Virchows Arch* 2004, 445(6):616-620.

24. Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA: Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* 1994, 368(6473):753-756.
25. Okamoto A, Demetrick DJ, Spillare EA, Hagiwara K, Hussain SP, Bennett WP, Forrester K, Gerwin B, Serrano M, Beach DH, *et al.*: Mutations and altered expression of p16INK4 in human cancer. *Proc Natl Acad Sci USA* 1994, 91(23):11045-11049.
26. Tam SW, Shay JW, Pagano M: Differential expression and cell cycle regulation of the cyclin-dependent kinase 4 inhibitor p16Ink4. *Cancer Res* 1994, 54(22):5816-5820.
27. World Health Organization Classification of Tumor: Pathology and Genetics. Tumors of the breast and female genital organs. Edited by: Tavassoli FA, Devilee P. IARC Press Lyon:260-289.
28. Rahmouni S, Cerignoli F, Alonso A, Tsutji T, Henkens R, Zhu C, Louis-dit-Sully C, Moutschen M, Jiang W, Mustelin T. Loss of the VHR dual-specific phosphatase causes cell-cycle arrest and senescence. Nat Cell Biol. 2006;8(5):524-31.
29. Robbins and Cotran (2004): Pathologic Basis of Disease, 7th Ed.Vol 3, Elsvier Publisher, New Delhi, India. P 269-288.
30. Slebos RJ, Lee MH, Plunkett BS, Kessis TD, Williams BO, Jacks T, et al. p53-dependent G1 arrest involves pRB-related proteins and is disrupted by the human papillomavirus 16 E7 oncoprotein. Proc Natl Acad Sci U S A 1994; 91: 5320-4.
31. Nam EJ, Kim YT. Alteration of cell-cycle regulation in epithelial ovarian cancer. Int J Gynecol Cancer 2008 Feb 19. (Epub ahead of print).
32. World Health Organization Classification of Tumor: Pathology and Genetics. Tumors of the breast and female genital organs. Edited by: Tavassoli FA, Devilee P. IARC Press Lyon:260-289.
33. Younis N, Khattab H, Zurayk H, el-Mouelhy M, Amin MF, Farag AM: A community study of gynecological and related morbidities in rural Egypt. Stud Fam Plann 1993, 24: 175-186

الملخص العربى

بحث دور فاكسينيا فيروس فى أتش 1 فى تطور سرطان عنق الرحم
بواسطة
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ان فاكسينيا فيروس والذى يمثل عن طريق الجين دى يو اس بى 3 قد تم ذكر دوره الحساس فى تطور دورة الخليه ولقد اثبتت الدراسات الحديثه ان نقصه يؤدى الى توقف النمو فى المرحلتين جى1 وجى 2 فى حالات سرطان عنق الرجم. ان اهميه بى 16 فى تشخيص سرطان عنق الرحم قد ذكرت فى العديد من ا لدراسات , ولقد تم تصميم هذه الدراسه بغرض تقييم دور فى اتش ار فى تطور سرطان عنق الرحم وايضا لمعرفه اهميه دور بى 16 كدلاله تستخدم فى التشخيص ثم تلى ذلك دراسة العلاقه بين العاملين, ولقد تضمنت الدراسه هذه الدراسه 10 حالات طبيعيه و12 حاله منخفضة الدرجه و18 حاله مرتفعه الدرجه و20 حاله سرطانيه ولقد تم استخدام الصبغه الهستوكيميائيه المناعيه لدراسه هذين البروتينين, ولقد اظهرت الدراسه ان ال فى اتش ار يوجد مراحل ماقبل الورم سواء المنخفضة او المرتفعه اما فى حاله السرطان فلقد وجد بوفره وخاصه فى النواه اما بى 16 فلقد اظهر تدرجا فى ازدياد فى الصبغه من الحالات منخفضة الدرجه الى الدرجه العاليه الى حالات السرطان , ان نتائج هذه الدراسة تقترح ان يكون ل ل قى اتش ار دور مبشر كدلاله فى سرطان عنق الرحم ويمكن ان يصبح هدف للعلاج, وبالنسبه للبى 16 فالنتائج تؤكد ماسبق ذكره من كونه دلاله تساعد على التشخيص.