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# EVOLUTION OF TUMOR SUPPRESSOR P16INK4A EXPRESSION IN CERVICAL CARCINOMA AMONG IRAQI WOMEN PATIENTS

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## ABSTRACT

**Background and objective:** p16INK4a (p16) is a tumor suppressor protein belongs to the family of INK4 a cyclin-dependent – kinase inhibitor. As overexpression of the protein p16 is correlated with the presence of high-risk HPV in malignant cervical lesions. This protein has been proposed as a surrogate marker of high-risk HPV infection in cervical cancer screening. Since high-risk viral DNA integration is necessary for neoplastic progression, we aimed to examine the expression of p16 in cervical cancer and determined any associated between presence of high risk of human papilloma virus and expression of p16 in cervial carcinoma among Iraqi women patients. **Method:** This study was carried out on 30 patients with hisopathologically confirmed primary cervical cancer in addition 20 cervical tissues from control individuals with no cancer as control groups. From each patients and control cervical tissues were proposed for chromogen in situ hybridization (CISH) techniques to investigation the presence of HPV (HPV 16 /18) DNA CISH in cervical tissues and for immunohistochemistry was used to detected expression of p16. **Results:** P16 expression was found in 28/ 30 (98%) tumor sample while negative cases in 2/30 (6%) tumor sample and no expression of p16 in cervical control groups. In this study demonstrated that all cervical carcinoma cases which positive for HPV (16/18) which represented 26/30 from the cases also positive for p16 expression (100%). **Conclusion:** Significant association obtained between p16 expression and high risk of HPV 16/18 CISH results in cervical carcinoma which indicate that inactivation of retinoblastoma protein (Rb) by E7 of HPV 16/18 among Iraqi women patients.

## INTRODUCTION

The p16INK4A protein is a cycline – dependent Kinase inhibitor that regulates the G1/S cell cycle check point by inactivating cycline D1-CDK4/6 complex activity and thereby enhancing retinoblastoma protein (PRb) activity and suppressing cell growth (Sherr & Robert, 2004). It was found that HPV – transformed cervical cancer are dependent on it expression and Knock down will lead to reduced proliferation (Mclaughlin *et al*., 2013).

A number of studies have demonstrated that p16 $^{INK4A}$ may be useful marker for squamous and glandular epithelial dysplasia in the uterine cervix (Klaes *et al*., 2001; Dray *et al*., 2005). Furthermore, over expression of p16$^{INK4A}$ appear to correlate with the degree of cervical neoplasia which may improve the histological diagnosis and hence the management of cervical lesion (Negri *et al*., 2004; Horn *et al*., 2008). Also It was reported that p16$^{INK4\ A}$ immunostaining can be used for discriminating integrated from non- integrated HPV infections (Dray *et al*., 2005; Arias *et al*., 2006).

The HPV – induced carcinogenesis is associated with low PRb protein level which lead to subsequent p16 upregulation and positive p16$^{INK4}$ immunostaining of HPV – associated tumors is 100% sensitive (Cunningham *et al*., 2006; Adelstein. J *et al*., 2009). The reports have been suggested that over expression of the p16$^{INK4}$ protein act as a surrogate biomarker of HPV – induced carcinoma (Koo *et al*., 2009). This study aimed to investigate expression of p16 in cervical cancer and determined any associated between presence of high risk of human papilloma virus (HPV) type (16/18) and expression of p16 in cervial carcinoma among Iraqi women patients.

## MATERIALS AND METHODS

In present study Cervical tissues were obtained from (30) patients with cervical cancer and (20) cases from individual cervical tissue were proved to be free from any significant pathological changes were considered as a negative control groups. Specimens belong to the period from April 2016 until March 2018. From each patient and control two blocks was taken formalin fixed, paraffin embedded cervical carcinoma.

Tumor, control blocks were collected from the archives of histopathology laboratories of Teaching Laboratories of the Medical City/Baghdad and Teaching Alkarmaa hospital, Teaching AlYarmouk hospital, AlWiya hospital for delivery as well as many private laboratories.

The diagnosis of these tissue blocks were based on the obtained pathological records of these cases from hospital files as well as histopathological laboratories records. A confirmatory histopathological re-examination of each obtained tissue blocks was done. 4 µm thick sections were made and adhesion on positively charged slides. Chromogenic in Situ Hybridization (CISH)/Detection system (Zytovisions GmbH. Bremerhaven. Germany) used to target DNA sequences using Digoxigenin– labelled long DNA probe for HPV types 16/18 in addition to Immunohistochemistry kit for detection p16(Cat. Number: AM 540-10M, Biogenex, U.S.A). Method was conducted according to the instructions of manufacturing companies leaflet.

Positive immunostaining for p16 protein include stain nucleus and cytoplasm of tumor cells as brown signal. positive tissue slide for P16 was pushed from (Biogenex, US) company. They include tissue from cervical squamous cell carcinoma that were previously known to contain the target marker. Quantification of P16 and p63 proteins expression were evaluated under light microscopy at X100, X400, and X1000. The counting of positive cells was performed at X1000.

The scoring for P16 was done as Semi-quantitative system that takes into consideration the proportion of positive cells (scored on a scale of 0-5) and staining intensity (scored on a scale of 0-3). Every tumor was given a score which represents the outcome of the summation of the intensity of the staining (intensity of score IS) (no staining = 0; weak = +1; intermediate staining = +2; strong staining = +3) with the percentage of stained cells(proportion score PS) score 0 = no stain, (score 1= less than 1%), (score 2; > 1-10%), (score 3; > 11-33%), (score 4; > 34-66%) and (score 5; > 66%). The proportion and intensity were then summed to produce total scores of 1 or 2 through 8. A score of 1 -2 was regarded as negative while 3-8 as positive.

The maximum score according to this system was (8) and scores of P16 according to Leong et al. (2004) include:- Score 0: 1-2, Score 1: 3-4, Score 2: 5-6, Score 3: 7-8. In this study Chi-square test was used to detect the significances between variables. All the statistical analysis was done by SPSS program (version-18). P-value was considered significant when < 0.05.

**RESULT**

In the current study, over expression of $p16^{INK4}$ was detected in (93.3%:28 out of 30) cells with cervical carcinoma, also cervical control group showed non $p16^{INK4}$ over expression. A

high percentage (40%: 12 out of 28 cases) involved cases of cervical cancer that improved as score (II), While (26.7%: 8 out of 28 cases) were found to have either score (I) or score (III). Statistically highly significant difference ($p<0.001$) were found in comparing the results according to score between the group of cervical carcinoma and cervical control group as seen in Table and figure (1).

**Table (1): Frequency distribution of immunohistochemistry results of $p16^{INK4}$ protein according to the signal scoring among study groups.**

| Study groups | Total $p16^{INK4}$ negative results | Positivity according to signal scoring | | | Total $p16^{INK4}$ positive results | p-value |
|---|---|---|---|---|---|---|
| | | Score I | Score II | Score III | | |
| Cervical Carcinoma (30 cases) | 2 (6.7%) | 8 (26.7) | 12 (40%) | 8 (26.7%) | 28 (93.3%) | <0.001 Highly Significant |
| Cervical Control (20cases) | 20 (100%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | |

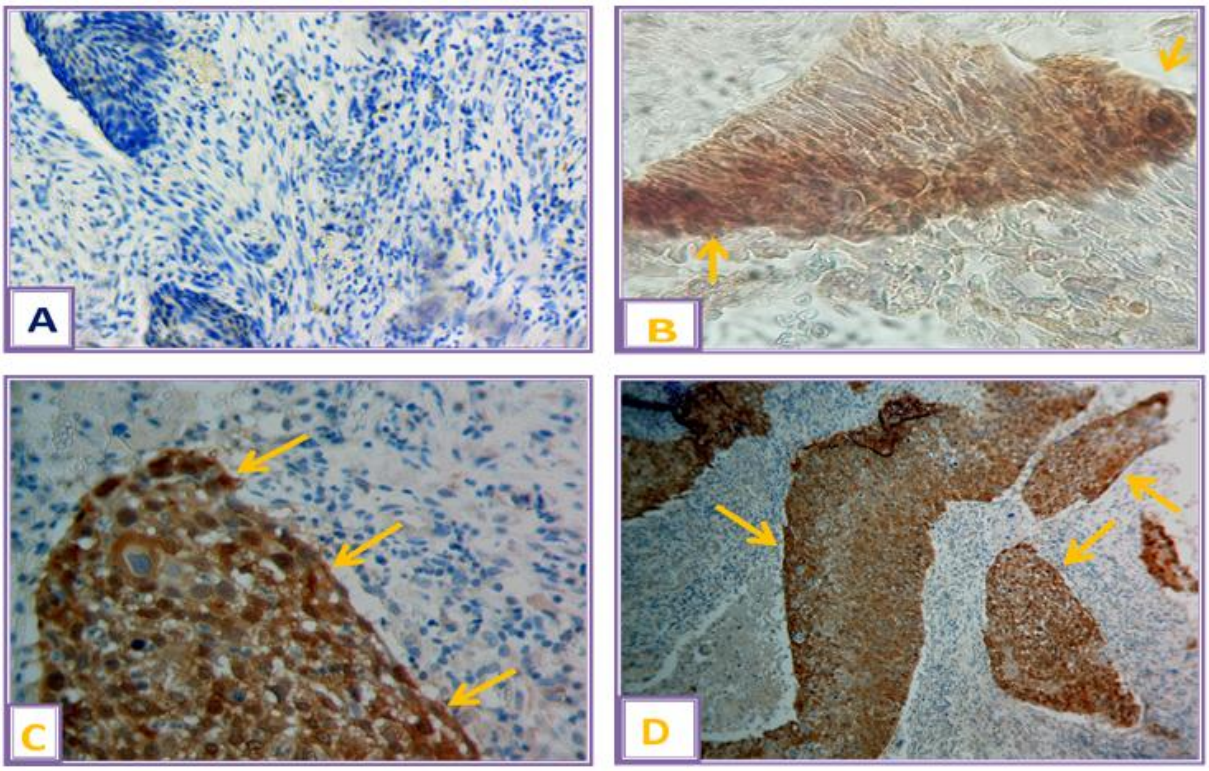


**Figure (1): Cervical Carcinoma Showing The Scoring Results of Immunohistochemical Staining of P16 Using Biotinylated -Labeled Anti- P16 Antibody Stained By Dab-Chromogen (Brown) And Counter Stained By Mayer's Hematoxyline Blue.**

**A:- Negative P16 – IHC Reaction (10x)**

**B:- Positive P16 – IHC Reaction With Low Score And Low Intensity Signal (40x)**

**C:- Positive P16 – IHC Reaction With Moderate Score And High Intensity Signal (40x)**

**D:- Positive P16 – IHC Reaction With High Score And High Intensity Signal (40x)**

On observing the total results of p16 – IHC in relation with the grading of the studied cases of cervical carcinoma, we notice that increasing percentage of cervical cancer cells that showed positive overexpression mutated $p16^{INK4A}$ protein along with the increasing of grading of cervical carcinoma, with some moderate different (Table 2).

In the current study as show in the Table (2) monitor that total p16 expression was detected in 28 /30 (93.3%) informative cervical carcinoma samples. It was detected that (88.8%: 8 out of 9 cases) of cervical carcinoma cases showed immunoreactivity for P16 have well differentiated grade, (94.4%: 17 out of 18 cases) of these tumor showed moderate differentiated grade, lastly (100%: 3 out of 3 cases) of these tumor showed poorly differentiated grade. The p16 seems to be related to the histological grade of cervical carcinoma and these findings appeared that increase expression of p16 with increase grading of cervical cancer. Non significant association was detected with different grades of malignant tumors ($p< 0.05$). (Table 2).

**Table (2): Correlation between p16 expression and histological grading among study groups.**

| Study groups | | | P16 expression | | p-value |
|---|---|---|---|---|---|
| | | | Negative | Positive | |
| Cervical carcinoma | Histological grades | Well | 1 (11.1%) | 8 (88.8 %) | 0.79 Non Significant |
| | | Moderate | 1 (5.5%) | 17 (94.4%) | |
| | | Poorly | 0 (0.0%) | 3 (100 %) | |
| | | Total | 2 (6.7%) | 28 (93.3%) | |

Several studies demonstrated that HPV positive tumors are characterized by high expression of $p16^{INK4A}$ protein (Agoff *et al*., 2003; Weinberger *et al*., 2006., Fakhry *et al*., 2008). Values of p16 immuno reactivity in HPV 16/18 positive and negative cervical carcinoma were demonstrated in Table (3). It was noticed that p16 overexpression was 26 (100%) in all cervical carcinoma cases which found to be positive for HPV16/18 CISH results, while 2(50%) of the cases which show negative for HPV16/18-CISH results also presented negative immunohistochemistry for p16 and 2(50%) of the cases which found to be negative for HPV16/18 CISH results showed positivity for p16 expression. Statistical comparison of p16 expression in HPV 16/18 positive and negative tumors demonstrated a significant results ($p< 0.05$).

**Table (3): Correlation between p16 expression and HPV16/ 18 CISH results among study groups.**

| Study groups | | | P16 expression | | Total | P-value |
|---|---|---|---|---|---|---|
| | | | Negative | Positive | | |
| Cervical Carcinoma | HPV | Negative | 2 (50%) | 2 (50%) | 4 (100%) | 0.013 Significant |
| | | Positive | 0 (0.0%) | 26 (100%) | 26 (100%) | |
| Endometrial Carcinoma | HPV | Negative | 4 (36.4%) | 7 (63.6%) | 11 (100%) | 0.534 Non Significant |
| | | Positive | 8 (42.1%) | 11 (57.9%) | 19 (100%) | |

## DISSCUSSION

In the current study it was found that a highly significant difference in the expression of $p16^{INK4}$ protein among cervical carcinoma and cervical control groups ($p<0.001$). The p16 protein expression was obviously higher in cervical carcinoma than in cervical control which is accordance with Milena *et al*., 2007; Kurshumliu *et al*., 2009; Lesnikova *et al.*, 2009; phaik *et al*., 2012.

In (2012) Izadi *et al*., detected over expression of $p16^{INK4}$ positivity in (90%) of squamous cell carcinoma and (91%) in high grade CIN2. This results show agreement with the results of the present study.

Keating and colleagues (2001) found that 70% of high squamous intraepithelial (HSIL) lesions had diffused strong expression of $p16^{INK4}$ protein while Godoye *et al*., (2008) demonstrated that $p16^{INK4}$ expression was made (48.3%) in the CIN1 as opposed to (94.4%) in the CIN2/CIN3 group showing a statistically significant difference between the two groups ($p_{value}$ = 0.001). Moreover, klaes *et al*, (2001) found in their study that (98%) of squamous cell carcinoma showed expression $p16^{INK4}$ while only (60%) of their CIN1 lesion had showed strong expression of $p16^{INK4}$. Possible reason of low frequency expression of $p16^{INK4}$ in low-grade lesions may due to a certain percentage are thought to be caused by low-risk HPV types because affinity of E7 protein of low –risk HPV types for Rb was much lower than that of high- risk HPV types, there would not be over expression of p16 $^{INK4}$ protein (Gage *et al*., 1990). Additionally, Keating *et al*.(2008) showed in their study that low-risk HPV is associated with less $p16^{INK4a}$ expression, and they suggest that different stages of high-risk HPV-induced cervical neoplasia may have different levels of $p16^{INK4a}$ expression.

In this respect, the $p16^{INK4}$ gene is inactivated by various genetic mechanisms including point mutation, deletions and hyper-methylation (Klase *et al*, 2001). In non- HPV associated tumors this inactivation lead to increased cyclin - dependent Kinase activity and inactivation

of Rb, however in HPV- associated tumors inactivation of Rb by E7 lead to markedly increased level of $p16^{INK4A.}$ (Marjoniemi, 2004). Some studies suggested that this process only occurs in cases of infection by high risk types of HPV. Thus p16 transcription may also be directly induced by the transcription factor E2F released from PRb after binding of viral oncoprotein E7 (Giarre *et al*., 2001).

According to the research of Tsoumpon *et al*. (2009) who observed that percentage of $p16^{INK4A}$ positivity of high grade squamous intraepithelial lesions varied between (44%) and (92%).

In the present study, Non significant association was detected with different grades of malignant tumors ($p< 0.05$). (Table 2) These results are consistent with other studies such as Geok *et al*., 2010; Jiaying *et al*., 2014 who reported that no significant relation between the alteration of p16 gene and grading of cervical carcinoma. Although this association did not reach statistical significance and a cytoplasmic overexpression pattern of p16 has been associated with progression in colorectal adenocarcinoma sequence and accelerated progression in breast cancer (Emig *et al*., 1998; Zhae *et al*., 2006).

In disagreement with our results Eun *et al*., 2008 who observed that strong p16 expression was associated with histologically poorly differentiated grade. However, recent studies reported that the p16 expression were higher in higher grade than in low grade (Keating *et al*., 2001; Lorenzato *et al*., 2005; Nam *et al*., 2007).

In contrast to our data, other reports showed that p16 expression was significantly associated with histological grades. The presence of heterogeneity between studies results from difference in many factors, including the age distribution, life style factor, use different p16 antibodies, and the standard of the IHC technique used particularly concerning the positivity threshold (Branca *et al*., 2004; Godye *et al*., 2005; Pabloconesa *et al*., 2009; Nerges *et al*., 2012).

Prospective and point studies have shown that p16 positive low grade lesions have high risk of progression than negative lesions, although this correlation was certainly not absolute (Negari *et al*., 2004; Harri *et al*., 2007). Ozaki *et al*., 2011 examined expression of $p16^{INK4A}$ biomarker in premalignant lesions to determine that these markers could help in predication

of the progression of CIN1 and found that expression of this biomarker was significantly higher in the progression group compared to the regression group being sensitive (86%).

In our study, it was show that all cervical carcinoma cases which found to be positive for HPV16/18 CISH results also show positive for p16 expression which represented (100%). This findings showed compatibility with Purushotham *et al*., 2014 who observed that all cervical carcinoma showed (100%) of p16 overexpression and (40%) of cervical carcinoma were positive for HPV16 genotypes.

In cervical lesion immunostaining for p16 and detection of HPV is already a diagnostic procedure. An increase expression of p16 determined by immunohistochemistry is a screening methods for the selection of patients for HPV subtyping (Benevolo *et al*., 2006; Missaoui *et al*., 2006).

In the current study, there was a significant association observed between p16 overexpression and HPV16/18 presence ($p_{value}$= 0.013). Our results were in accordance with Nicholas *et al*., 2003; Hirouki *et al*., 2009; Marcia *et al*., 2005; Godoy *et al*., 2008; Jin, 2008; Jung, 2010.

In high risk – HPV positive cervical cancer, the oncogene E7 disrupts pRb / E2F(E2 Factor) interaction, releases active E2F and induce the pRb degradation (Nehls, 2008). The existence of the regulatory feed back in the pRb / p16 pathway lead to overexpression of p16 in cervical tumors (Ivanova,. 2007).

Based on studies published so far, p16 could potentially be utilized in the detection of HR-HPV in pap smears. many reports have described the successful application of p16 immunohistochemistry (IHC) to liquid based and conventional pap smears, with good concordance between p16 and pap smear results (Biobbo *et al*., 2002), in addition Ekalaksanan *et al*., 2006, stated that all cases of HSIL and Squamous cervical carcinoma in his research showed p16 positive by IHC.

Maria *et al*., 2006, who stated that, in this context, p16 testing can substantially improve the conventional morphological diagnosis of cervical preneoplastic lesions. However, morphological criteria alone are not sufficient to distinguished lesions that may regress from those that might progress and persist, therefore, the evolution of HR- HPV infection as well as p16 immunoreactivity, could be useful tools of particular clinical value in identifying cases with higher possibility to progress to high grade lesions. Tumors that lack HR-HPV positivity

may have a larger number of mutations in genes coding for cell cycle regulating proteins to be transformed, and thus may be more therapy resistant. This is corroborated by the fact that HR-HPV positive carcinoma generally is more susceptible to radiochemotherapy than HPV-negative counterparts.(Fakhry *et al*., 2008).

Iana *et al*., 2009 conclude that in larger numbers of section we were able to proven that immunohistochemical detection $p16^{INK4A}$ expression can be used as a specific diagnostic marker of all degrees of cervical dysplasia and cervical cancer and possibly as a surrogate marker for HPV infection, due to the relationship between $p16^{INK4A}$ and HPV E7 inactivated Rb protein.

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