# Changes in p16$^{INK4}$ Protein Expression During Cervical Cancer Progression and Correlation to HPV Infection

*Arwa Al-Shwaikh, \*[1]Ismail Latif, [1]Saad Kadhim[1]*

## Abstract

**Aim:** Recent studies have shown that the protein level of cell cycle regulator p16 is overexpressed in high-risk HPV-related squamous cell carcinomas as well as their precursor lesions. The aim of this study was to compare the different p16 immunoexpression patterns in different cervical lesions in relation to HPV status.

**Materials and Methods:** A total of 80 cervical tissue samples were included in this study. Seventy archival tissue biopsy samples comprised a risk group for HPV infection and/or cervical neoplasia; these were selected from the histopathology files of Al-Kadhimiya Teaching Hospital, Al-Ulwiya Teaching Hospital, Al-Yarmouk Hospital, the Medical City Department of Teaching Laboratories, and four private laboratories. The remaining 10 normal postmortem tissue biopsies were obtained from the Institute of Forensic Medicine and considered as a control group. Immunohistochemistry was done for detection p16.

**Results:** A significant correlation was found between p16 expression and the progression of the disease ($p < 0.01$). A significant difference ($P < 0.01$) was found in the mean of p16 immunostaining between HPV-positive and negative groups in CIN II/III and ISCC.

**Conclusion:** This study demonstrated that p16 immunohistochemical evaluation could be useful as a biomarker for the progressive malignancy in cervical lesions.

**Keywords:** Uterine cervix, p16, IHC, HPV.

## Introduction

p16 $^{INK4a}$ is a tumor suppressor protein involved during the G1/S phase. This protein, encoded by the CDKN2A (INK4A) tumor suppressor gene on chromosome 9 p21, inactivates the function of cdk4- and cdk6-cyclin D complexes.[1] Functional loss of p16 has been reported for many human cancers whereas in the Human Papilloma Virus (HPV)- associated cervical carcinomas, p16 overexpression has been observed.

The HPV E7 gene product binds to the hypophosphorylated form of the Retinoblastoma (Rb) family of proteins.

This binding results in the phosphorylation of these proteins, in their enhanced degradation by ubiquination, and in the release of the transcription factors of the E2F family activating the transcription of gene regulating cell proliferation.[2]

1. **Al-Nahrain University, Iraq.**
 *\* Correspondence should be addressed to:*
 **Arwa Al-Shwaikh**

As the HPV E7 oncogene product inhibits the activity of the Rb protein, p16 is up-regulated via the loss of the negative feedback control of the pRb expression.[3,4] The p16 transcription may also be directly induced by the transcription factor E2F released from pRb after the binding of the viral oncoprotein E7.[5]

## Materials and Methods

***Tissue Samples:*** A total of 80 tissue samples from uterine cervices were included in this study. All samples were known HPV status by *in situ hybridization* (ISH) technique (using high spectrum probe). Seventy out of 80 samples that comprised a risk group in our study were obtained from archival paraffin embedded blocks selected from the histopathology files of Al-Kadhimiya Teaching Hospital, Al-Ulwiya Teaching Hospital, Al-Yarmouk Hospital, Medical City Department of Teaching Laboratories, and from four private laboratories. The remaining 10 samples (autopsies) which comprised a normal control group were obtained from the Institute of Forensic Medicine. These autopsies were taken from virgin female cervices based on the fact that HPV infections are practically non-existent in the celibate population.[6] Ethical approval for use of all specimens was obtained and the histopathologic diagnosis was confirmed by the review of freshly prepared hematoxylin and eosin-stained slides by certified pathologists and classified according to criteria outlined by the World Health Organization.

***Materials:*** p16 Research Kit, code number OA315 (DakoCytomation, Denmark).

***Quality Control:*** Paraffin-embedded tissue sections containing colon cancer were used as a positive control for p16.[7] The negative control was obtained by replacing the primary Ab with culture supernatant containing monoclonal mouse IgG2a anti-body to *Aspergillus niger* glucose oxidase, an enzyme which is neither present nor inducible in mammalian tissues (DakoCytomation, Denmark). Both positive and negative control was included for each run of immunohistochemisty.

***Procedure of Immunohistochemistry (IHC):*** The procedure of immunohistochemical was carried out in accordance with the manufacturer's instructions (DakoCytomation, Denmark). The process included: baking the slides in a hot air oven at 60 °C overnight and then washing in xylene. After gradual hydration through graded alcohols, the sections were rinsed in distilled water. After that the antigen retrieval [8,9] and endogenous peroxidase quenching were done. For localization of p16 protein, the sections were incubated for 60 min at 37 °C with a 1:25 dilution of the monoclonal antibody of p16 and then placed at 4 °C overnight. After that the slides were visualized by the visualization reagent and incubated at 37 °C for 30 min. Next, 100 μl freshly prepared DAB-substrate chromogen solution was placed onto the section and incubated in darkness at room temperature for 10 min. Then the slides were rinsed gently with wash buffer and counterstained with Mayer's hematoxylin, dehydrated, cleared and mounted.

***Evaluation of the Immunostaining:*** Slides were examined by light microscope at X400 magnification. Strong nuclear as well as cytoplasmic staining was considered a positive score on a semi-quantitative scale, as follows: negative (< 5% of cells were positive), focally positive (≤ 25%) and diffuse positive (> 25%).[10]

***Statistical Analysis:*** A spearman correlation was done to determine any association between the p16 expression and the disease progression. Comparison between the quantitative immunohistochemical expression of p16^{INK4} in relation to the HPV status was made using a t-test. All analysis was performed using the SPSS program. A $p$ value of less than 0.05 was considered statistically significant.

## Results

Immunohistochemical analysis for p16 was performed in all 80 specimens investigated in this study, and the results in relation to histological diagnosis and HPV status are summarized in

table (1). In all normal and condylomatous changes, cervical tissue as well as normal tissue fragments identified in specimens with cervical intraepithelial neoplasia (CIN) did not show immunohistochemically detectable p16 expression. In a few cases, single isolated epithelial cells considered negative within reactive lesions or endocervical glands showed positive staining (figure 1).

In CIN I cervical lesions, either no (4/9, 44.4%) or only weak focal p16 staining was seen (5/9, 55.5%). In CIN II/III, p16 was focal (8/14, 57.1%) and intensities varied from weak to moderate. However, strong and diffuse immunoreactivity for p16 was uniformly observed in both the nuclei and cytoplasm (2/14, 14.3%) of CIN II/III which they are also HPV-positive. Strong and diffuse immunostaining for p16 was seen in all ISCC infection with HPV. On other hand, 5 of the 18 HPV-negative cancer cases also demonstrated strong and diffuse p16 protein immunoreactivity and the remaining 13 cases showed focal to negative staining for p16. Expression of p16 was generally very strong in dysplastic and neoplastic epithelia and clearly distinguished these cells from adjacent normal epithelia or stromal cells (figure 1).

Spearman's correlation test revealed a highly significant relation ($p < 0.01$) between p16 staining and the increasing grade of squamous dysplasia. The results of the present study evidenced that p16 is a useful marker for squamous cell carcinoma of the cervix uteri and its precursors. The t-test showed that there is a significant difference (t = 8.192, $p < 0.01$) in the mean of p16 immunostaining between HPV-positive and negative groups as shown in table (2).

**Table (1): Quantitative immunohistochemical expression of p16$^{INK4}$ in relation to histological types and HPV status.**

| Histological Type* & HPV status | No. | p16$^{INK4}$ staining ( % ) * | | | |
| | | Negative (< 5) | Focal ( ≤ 25 ) | Diffuse ( > 25 ) | Total |
|---|---|---|---|---|---|
| **Normal** | 10 | 10/10 (100) | 0/10 (0) | 0/10 (0) | |
| HPV-positive | 0 | 0/10 ( 0 ) | 0/10 (0) | 0/10 (0) | 0/10 (0) |
| HPV-negative | 10 | 10/10 (100) | 0/10 (0) | 0/10 (0) | |
| **Condylomatous changes** | 20 | 20/20 (100) | 0/20 (0) | 0/20 (0) | |
| HPV-positive | 6 | 6/20 (30) | 0/20 (0) | 0/20 (0) | 0/20 (0) |
| HPV-negative | 14 | 14/20 (70) | 0/20 (0) | 0/20 (0) | |
| **CIN I** | 9 | 4/9 (44.4) | 5/9 (55.55) | 0/9 (0) | |
| HPV-positive | 1 | 1/9 (11.1) | 0/9 (0) | 0/9 (0) | 5/9 (55.5) |
| HPV-negative | 8 | 3/9 (33.3) | 5/9 (55.55) | 0/9 (0) | |
| **CIN II/III** | 14 | 4/14 (28.5) | 8/14 (57.1) | 2/14 (14.3) | |
| HPV-positive | 3 | 0/14 (0) | 1/14 (7.1) | 2/14 (14.3) | 10/14 (71.42) |
| HPV-negative | 11 | 4/14 (28.5) | 7/14 (50) | 0/14 (0) | |
| **ISCC** | 27 | 4/27 (14.8) | 9/27 (33.33) | 14/27 (51.9) | |
| HPV-positive | 9 | 0/27 (0) | 0/27 (0) | 9/27 (33.3) | 23/27 (85.1) |
| HPV-negative | 18 | 4/27 (14.8) | 9/27 (33.33) | 5/27 (18.5) | |

*\* Spearman's rho=0.807, p < 0.01.*

**Table (2): Difference in the mean of p16 immunostaining between HPV-positive and negative groups in CIN II/III and ISCC cases.**

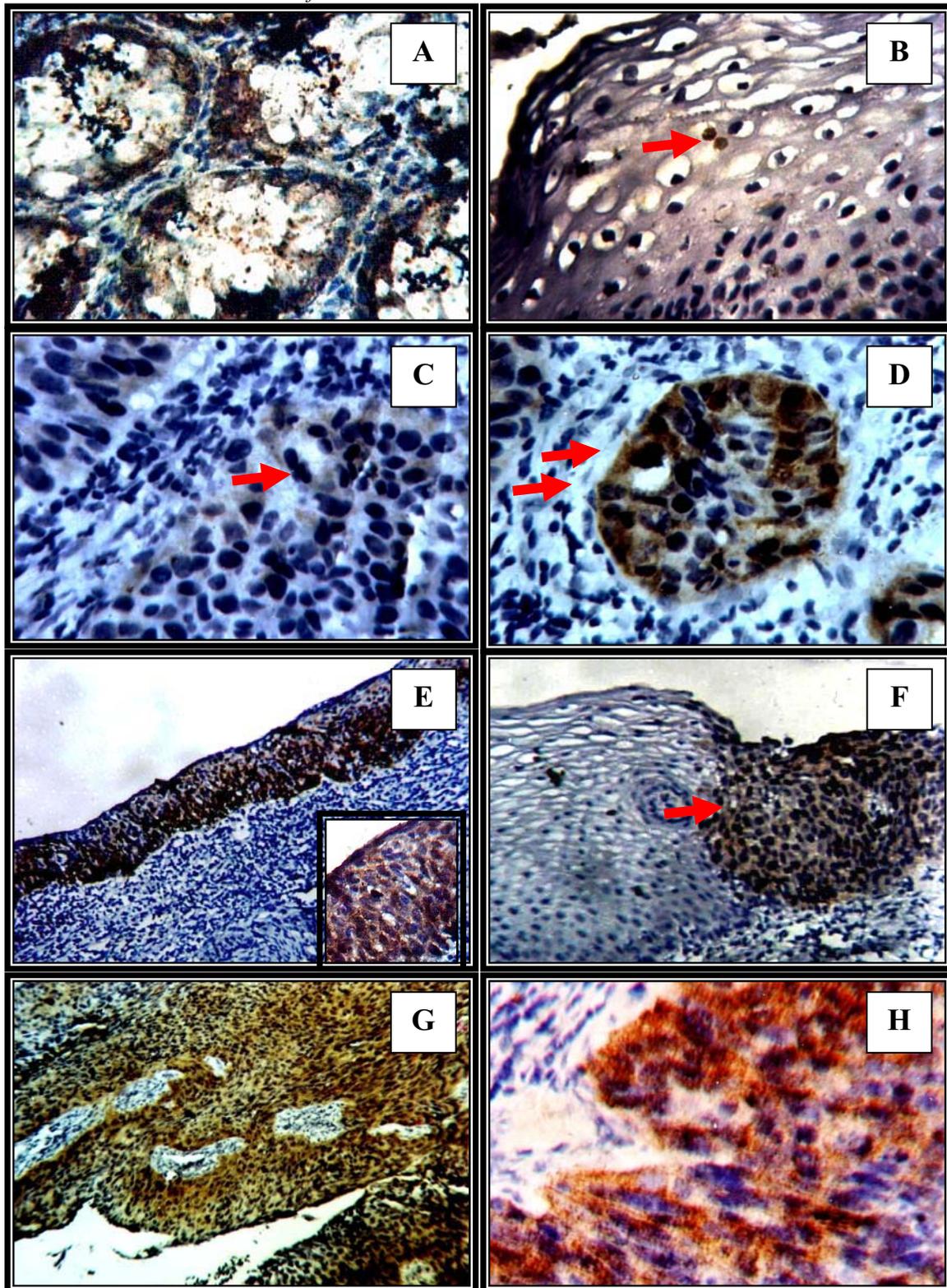| HPV status | NO. | Mean of p16$^{INK4a}$ immunostaining ± S.D.* | Statistical analysis |
|---|---|---|---|
| HPV-positive | 12 | 72.50 ± 22.953 | t = 8.192 |
| HPV-negative | 29 | 18.41 ± 7.559 | P < 0.01 |

*\* S.D.: Standard deviation.*

***Figure (1): Immunohistochemical staining of p16 protein. Staining by DAB chromogen (dark brown), counterstained with Mayer's haematoxylin. [A] Colorectal carcinoma, positive control for p16$^{INK4a}$ (X400). [B, C, D] Scoring system for p16$^{INK4a}$ immunostaining (X400): [B] Mild condylomatous changes with p16$^{INK4a}$ staining in only two isolated cells (red arrow), this case was judged as negative; [C] ISCC with p16$^{INK4a}$ staining in ≤ 25% of tumor cells (focal pattern); [D] >25% of tumor cells (diffuse pattern); no positive staining was seen in the stroma (red arrows). [E, F] CIN III lesions: [E] with diffuse p16$^{INK4a}$ staining throughout the full thickness of the epithelium X100 (inset, X250); [F] the borderline between positive and negative staining (X250). [G] ISCC with diffuse and intense staining corresponding to the HPV-infection (X100). [H] Higher magnification of [G] (X400).***

## Discussion

The *p16* gene is frequently inactivated in a variety of malignant tumors by deletion, point mutation or promoter hypermethylation; no gene alterations were detected in most studies on primary cervical carcinomas or cervical cancer cell lines indicating that they are not required for the development of this tumor type.[11] Contrary results, however, were reported by Kim et al,[12] who found a high percentage (70%) of p16 mutation in cervical cancer specimens. However, instead of down-regulation, marked overexpression of the *p16* gene was demonstrated by a strong and diffuse immunostaining in precancerous and malignant cervical lesions, which are infected with high-risk HPV types.[5]

Table (1) shows the data of p16 IHC expression in relation to histopathological diagnosis and HPV status in cervical biopsies. Our own results showed that the frequency of p16 immunostaining (0%, 0%, 55.5%, 71.42% and 85.1% in normal, condylomatous changes, CINI, CINII/III and invasive squamous cell carcinoma ISCC, respectively) in formalin - fixed, paraffin embedded cervical tissue might be nearly the same as those reported by other studies.[13,7] The value of p16 as a diagnostic marker of cervical dysplasia and carcinomas of cervix uteri has already been demonstrated.[7,10,11,14,15] Similarly, we found a highly significant correlation between p16 staining and the extent of histological abnormality ($p < 0.01$).

The reaction was always evident and the marked lesion easy to distinguish from reactive changes. Some studies on cervical cancer have demonstrated that p16 may serve as a marker to differentiate neoplastic lesions from hyperplastic or reactive lesions.[11] In the present study, p16 positivity patterns were found to correlate with the stage of tumor progression, focal and diffuse features being associated, respectively, with premalignant and malignant cervical lesions, and negative labeling with benign lesions. These results showed a strong correlation between overexpression of p16 protein and cervical lesions that were malignant or with high malignancy potential.

Interestingly, lesions with HPV-positive, but p16-negative, were diagnosed as condylomatous changes and CINI, whereas those combining HPV DNA with diffuse p16 expression were classified as CINII/III and ISCC, respectively as shown in table 1. These results also demonstrate that different patterns of p16 immunopositivity could be associated with low- or high-risk HPV infection in cervical tissue. Begum et al [16] found that only strong, diffuse staining of the cytoplasm and nuclei, and not focal or weak staining, is associated with the presence of high-risk HPV in neoplasia of the female genital tract.

Since expression of p16 underlies a negative feedback control through pRb, reduction or loss of the pRb function should result in enhanced p16 levels in the respective cells. Taken together, these data suggest that inactivation of pRb through HPV E7 results in the enhanced expression of p16, which might therefore represent a specific and sensitive biomarker for cells with active expression of HPV oncogenes.[17,14]

Several studies [13,18] suggested that the use of p16 immunohistochemistry may identify low-grade cervical lesions with increased risk for progression to high-grade precancer or invasive lesions because progression from low-grade to high-grade precancer is very strongly associated with the presence of high-risk HPV-DNA squences.

Also, Murphy et al [15] found a strong correlation between HPV and p16 positivity, although it should be noted that p16 expression was also seen in a limited number of HPV-negative cases. However, we found focal p16 expression in cervical lesions that were negative for HPV DNA, of which 5/9 (55.5%) were diagnosed as CINI, 7/14 (50%) as CINII/III and 9/27 (33.3%) of ISCC. This result suggests that moderate expression in the absence of viral DNA could be associated with histological types and that a non-HPV E7 mediated mechanism of p16

upregulation may exist. Loss of transcriptional repression in the presence of inactivating mutations in the *Rb* gene is the most well defined non-HPV related mechanism of p16 upregulation. Indeed, p16 expression may in some cases be independent of pRb.[11] The t-test showed that there is a significant difference ($P < 0.01$) in the mean of p16 immunostaining between HPV-positive and negative groups in CIN II/III and ISCC, and as shown in table 2.

On other hand, five of the 18 HPV-negative ISCC cases also demonstrated strong and diffuse p16 protein immunoreactivity which may have been due to false negative results for HPV DNA, by a very low HPV copy number that was below the limit of detection by our ISH method, or by the presence of a novel unknown HPV subtype.[17]

In summary, routine use of p16$^{INK4a}$ immunostaining in cervical biopsies can reduce interobserver disagreement in the interpretation of cervical lesions and lead to a more accurate detection of high-grade precancerous lesions and CINI associated with progressive high-risk HPV infection. p16$^{INK4a}$ immunostaining directs pathologists' attention to the intensely stained areas in the slides and is particularly helpful in detecting small dysplastic lesions. This method, applied to cytology, may also prove to be useful as an alternative to HPV DNA testing for the triage of low-grade smear abnormalities or in primary screening.[13]

### References

1. Klussmann JP, Gultekin E, Weissenborn SJ, Wieland U, Dries V, Dienes HP, Eckel HE, Pfister HJ, Fuchs PG. Expression of p16 protein identifies a distinct entity of tonsillar carcinomas associated with human papillomavirus. American Journal of Pathology, 2003; 162 (3): 747 – 753.
2. Webster K, Parish J, Pandya M, Stern PL, Clarke AR, Gaston K. The human papillomavirus (HPV) 16 E2 protein induces apoptosis in the absence of other HPV protein and via a p53-dependent pathway. The Journal of Biological Chemistry, 2000; 275 (1): 87 – 94.
3. Natarajan E, Saeb M, Crum CP, Woo SB, Mckee PH, Rheinwald JG. Co-expression of p16$^{INK4A}$ and lamini 5γ2 by microinvasive and superficial squamous cell carcinomas in vivo and by migrating wound and senescent keratinocytes in culture. American Journal of Pathology, 2003; 163 (2): 477 – 491.
4. Sahebali S, Depuydt CE, Segers K, Moeneclaeg LM, Vereecken AJ, March EV, Bogers JJ. p16$^{INK4a}$ as adjunct marker in liquid-based cervical cytology. Int. J. Cancer, 2004; 108: 871 – 876.
5. Milde-Langoshch K, Riethdorf S. Role of cell - cycle regulatory proteins in gynecological cancer. J. Cell. Physiol, 2003; 196: 224 – 244.
6. Menczer J. The low incidence of cervical cancer in Jewish women: has the puzzle finally been solved. I.M.A.J. 2003; 5: 120 – 123.
7. Wang JL, Zheng BY, Li XD, Angstrom T, Lindstrom MS, Wallin KL. Predictive significance of the alteration of p16$^{INK4A}$, p14 ARF, p53, and proliferating cell nuclear antigen expression in the progression of cervical cancer. Clinical Cancer Research, 2004; 10: 2407 – 2414.
8. Cook DJ. Exfoliative cytology and related techniques and Immunological techniques. In: Cellular pathology. Palliste, C.J. (editor). Butterworth Heinemann. Oxford Auckland Boston Johannesburg Melbourne New Delhi, 2000; Chapter 10: p.190 and Chapter 12: PP. 209 – 237.
9. Miller RT. Technical immunohistochemistry: achieving reliability and reproducibility of immunostaions. Propath. Laboratory, Inc., 2001; (Available online at http:// www.propathlab.com ).
10. Negri G, Vittadello F, Romano F, Kasal A, Rivasi F, Girlando S, Mian C, Egarter-Vigl E. p16$^{INK4a}$ expression and progression risk of low-grade intraepithelial neoplasia of cervix uteri. Virchows Arch, 2004; 445: 616 – 620.
11. Fregonesi PAG, Teresa DB, Duarte RA, Neto CB, De-Oliveira MRB, Soares CP. p16$^{INK4A}$ immunohistochemical overexpression in premalignant and malignant oral lesions infected with human papillomavirus. The Journal of Histochemistry and Cytochemistry, 2003; 51 (10): 1291 – 1297.
12. Kim JR, Kim SY, Kim MJ, Kim JH. Alterations of CDKN2 (MTS/p16INK4A) gene in paraffin - embedded tumor tissues of human stomach, lung, cervix and liver cancers. Experimental and Molecular Medicine, 1998; 30 (2): 109 – 113.

13. Klaes R, Benner A, Friedrich T, Ridder R, Herrington S, Jenkins D, Kurman RJ, Schmidt D, Stoler M, Doeberitz M. p16$^{INK4a}$ immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. The American Journal of Surgical Pathology, 2002; 26 (11): 1389 – 1399.

14. Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U, Dallenbach-Hellweg G, Schmidt D, Doeberitz MVK. Overexpression of p16$^{INK4A}$ as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. Int. J. Cancer, 2001; 92: 276 – 284.

15. Murphy M, Ring M, Heffron C, King B, Killalea A, Hughes C, Martin C, McGuinness E, Sheils O, Leary J. p16$^{INK4A}$, CDC6, and MCM5: predictive biomarkers in cervical cancer. J. Clin. Pathol, 2005; 58: 525 – 534.

16. Begum S, Gillison ML, Ansari-Lari MA, Shah K, Westra WH. Detection of human papillomavirus in cervical lymph nodes: a highly effective strategy for localizing site of tumor origin. Clinical Cancer Research, 2003; 9: 6469 – 6475.

17. Sano T, Oyama T, Kashiwa-bara K, Fukuda T, Nakajima T. Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions. American Journal of Pathology, 1998; 153 (6): 1741-1748.

18. Miller RT. Immunohistochemistry utility of p16 (INK4a) immunostaining in cervical biopsies. 2002 (Available online at www.propathlab.com ).

# التغير في تعبير بروتين p16<sup>INK4</sup> خلال تطور وتقدم سرطان عنق الرحم وعلاقته بالفايروس الحليمي البشري

أروى مجاهد عبد الله الشويخ،[1] اسماعيل ابراهيم لطيف،[1] سعد كاظم[2]

1–فرع الأحياء المجهرية، كلية الطب، جامعة النهرين، العراق؛ 2–فرع علم الأمراض والطب الشرعي (العدلي)، كلية الطب، جامعة النهرين، العراق

**الخلاصة:**

أظهرت دراسات عديدة زيادة في تعبير البروتين المنظم لدورة الخلية p16 في سرطان عنق الرحم المحرشف وحالات سوء النمو الظهارية مختلفة الدرجات. أن الهدف من هذه الدراسة هو تحديد ما إذا كان انتاج هذا البروتين ذا فائدة للاستدلال على تطور وتقدم المرض وعلاقتة مع وجود الفايروس.

تضمن البحث ثمانين عينة، منها سبعون عينة أخذت من الأنسجة الأرشيفية المطمورة في شمع البارافين والتي تمثل مجموعة الخطر المحتوية على دلائل تشير إلى وجود إصابة بالفايروس الحليمي البشري مع/ أو وجود حالات سوء نمو ظهارية (السرطانية أو غير السرطانية) وقد اختيرت العينات من ملفات مختبرات التحليلات النسيجية من مستشفى الكاظمية التعليمي، مستشفى العلوية، مستشفى اليرموك التعليمي، المختبرات التعليمية في مستشفى مدينة الطب وبعض المختبرات الخاصة. وتمثل العشر عينات المتبقية خزعات مأخوذة بعد الوفاة من عنق أرحام نسوة أبكار كمجموعة سيطرة اثبت الفحص النسيجي عدم وجود عوارض أو إصابات فيها. لقد تم التحري عن تعبير بروتين p16 بطريقة التصبيغ الكيميائي النسيجي المناعي (IHC) Immunohistochemistry.

ان نتائج تصبيغ بروتين p16 بينت أن هناك علاقة ذات مغزى ($p < 0.01$) مع تطور وتقدم المرض كما ان تصبيغ تصبيغ p16 مرتبطا بعلاقة ذات مغزى ($p < 0.01$) مع وجود الفايروس الحليمي البشري. لقد اظهرت النتائج ان تقييم مدى تعبير بروتين p16 ذات فائدة للاستدلال على تطور وتقدم الورم في ظهارة نسيج عنق الرحم.

<u>**الكلمات الدالة**</u>: نسيج عنق الرحم، p16, IHC, HPV .