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Evaluation of P16, Human Papillomavirus Capsid Protein L1 and Ki67 in Cervical Intraepithelial Lesions: Potential Utility in Diagnosis and Prognosis

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KEYWORDS

P16; HPV L1; KI67; CIN; Cervical Carcinoma.

SUMMARY

Purpose: Cervical dysplasia, potentially precancerous lesion, has increased in young women. Detection of cervical dysplasia is important for reducing morbidity and mortality in cervical cancer. This study analyzes the immunohistochemical expression of p16, HPV L1 capsid protein and Ki67 in cervical intraepithelial lesions and correlates them with lesion grade to develop a set of markers for diagnosis and detect the prognosis of cervical cancer precursors.

Materials and methods: 75 specimens were analyzed including 15 cases CIN 1, 28 CIN 2, 20 CIN 3, and 12 cervical squamous carcinoma, besides 10 normal cervical tissues. They were stained for p16, HPV L1 and Ki-67. Sensitivity, specificity, predictive values and accuracy were evaluated for each marker.

Results: p16 expression increased during progression from CIN 1 to carcinoma. HPV L1 positivity was detected in CIN 2 and decreased gradually as the CIN grade increased but disappear in carcinoma. Strong Ki-67 expression was observed in high grades CIN and carcinoma. p16, HPV L1 and Ki67 were sensitive but with variable specificity in detecting CIN lesions.

Conclusions: p16, HPV L1 and Ki67 are useful markers in establishing the risk of high-grade CIN. They complete each other to reach accurate diagnosis and detecting the prognosis.

INTERODUCTION

Cervical carcinoma is the second most common malignancy in women worldwide [1]. World Health Organization **(WHO)** introduced cervical cancer as the first cancer which is entirely caused by infection [2]. It has a multi-step carcinogenic progression from low through moderate to high-grade epithelial lesion. Detection and treatment of cervical dysplasia in young women is important for reducing morbidity and mortality [3]. Cervical biopsies, in conjunction with Pap cytology testing, Human Papillomavirus **(HPV**) DNA testing, and colposcopy, have an important role in the evaluation and management of women with cervical dysplastic lesions, which is crucial for the prevention and early detection of cervical cancer [4].

Cervical Intraepithelial Neoplasia **(CIN)** has been classified into 3 grades by WHO: grade 1, mild dysplasia (CIN 1); grade 2, moderate dysplasia (CIN 2); and grade 3, severe dysplasia with carcinoma in situ (CIN 3) [5]. CIN has been classified into low grade Squamous Intraepithelial Lesion **(SIL)** and high-grade SIL in the Bethesda classification system [6]. CIN 1 is equivalent to low-grade SIL category, while CIN 2 and CIN 3 are equivalent to high-grade SIL. According to the guidelines of the American Society of Colposcopy and Cervical Pathology **(ASCCP),** CIN 1 patients should be followed up without treatment because most cases of CIN 1 regress spontaneously. The guidelines recommend that women with CIN 2/3 should undergo an excisional treatment. Although these treatments are efficacious in preventing cervical cancer, they also have been associated with pregnancy complications, such as cervical stenosis or incompetence. Recently, several biomarkers have been evaluated for their potential role to improve the diagnostic accuracy of cervical biopsy [4].

High-risk Human Papilloma Virus **(HPV)** infection is known to be the most important event in the malignant transformation of cervical epithelium. The life cycle of HPV infection consists of 2 phases. The first phase is a ‘‘productive’’ phase. In this phase, HPV infects basal keratinocytes. Then the early HPV genes E1, E2, E5, E6, and E7 are expressed, and the viral DNA replicates. The productive phase refers to the early stage of HPV infection with L1 capsid protein expression. L1, or the major capsid protein, together with L2, the minor capsid protein is produced within the cytoplasm and translocated into the nucleus. The presence of HPV L1 capsid protein within the dysplastic cells is proof of a completed HPV life cycle. HPV L1 capsid protein–negative cases, however, have lost the ability to produce virions [7]. In contrast, the second phase is the ‘‘transformation’’ phase, in which HPV DNA is integrated into the host DNA. Persistent cervical infection and integration with oncogenic HPVs are the most important risk factors for progression from low grade SIL to high grade SILs and development of squamous carcinoma [8]. The number of cervical cancer cases is, however, small compared with the number of women infected with HPV. Therefore, the main problem is how to identify individuals who are at risk of progressive disease among the large number of individuals infected with HPV [9]

E6 and E7 oncoproteins from HPV inhibit the tumor suppressor functions of p53 and Rb protein, respectively [10]. The E7 oncoprotein has been shown to bind to Rb protein, resulting in E2F over expression that leads to inhibition of cyclin D1–dependent kinase activity and, consequently, induces expression of a p16-related transcript [11]. Viral oncongenes of low-risk HPV have no effect on p16, because the affinity of low-risk HPV E7 protein for cellular Rb is 10-fold lower than that of high risk HPV E7 for Rb [12]. In other words, increased expression of p16 indicates that there is HPV-induced abnormality of the cell cycle and therefore immortalization of HPV infected cells [13]. Although p16 immunostaining has been correlated with the severity of abnormalities in cervical lesions, variations has resulted in uncertainty for the analysis of p16 levels. This dilemma is underscored by the fact that p16 ex-pression can be up regulated in non-dysplastic cervical lesions [14], so evaluation of p16 staining requires additional morphologic evaluation. Other biomarkers have been proposed for use in triaging women with cervical dysplasia to increase diagnostic accuracy, such as Ki-67 [15].

Ki-67, a cell proliferation marker, is expressed during all phases of the cell cycle except G0 [1]. Ki-67 and p16 are complementary alternative biomarkers for HPV-related cervical neoplasia [13].

The goal of this study is to analyze the immunohistochemical expression of p16, HPV L1 capsid protein and Ki-67 in cervical intraepithelial lesions and correlate them with lesion grade and differentiate them from benign lesions to develop a more effective set of surrogate markers for the prediction of high-risk precursor or invasive cervical lesions.

MATERIALS AND METHODS

Case Selection and Sample Preparation

A total of 75 specimens of formalin-fixed, paraffin-embedded cervical tissues were analyzed. They were obtained from the archive files of the Department of Pathology at the Tanta University Hospital from 2009-2013. The punches were 1 mm in diameter and consisted of two cores with full thickness of the cervical epithelium. Clinical information was obtained from the patients' medical records. Cases comprised 15 cases of CIN 1, 28 cases of CIN 2, 20 cases of CIN 3, and 12 cervical squamous carcinoma. Cases of cervical squamous carcinoma were classified and graded according to WHO criteria and staged according to criteria of the International Federation of Gynecolology and Obstetrics **(FIGUREO)** [5]. Besides, 10 normal cervical tissues obtained from hysterectomy specimen.

Immunohistochemical Analysis

For the study, 4-µm-thick serial sections of formalin fixed, paraffin-embedded tissue were cut and mounted on positively charged glass slides. After incubation at 60°C overnight and deparaffinization, the tissue sections underwent heat retrieval for 20 minutes with Tris-EDTA buffer (Thermo Fisher Scientific, Waltham, MA) for p16. Sections were stained using the

following primary antibodies: anti-p16 (clone 16P07, 1:40 dilution; mouse monoclonal antibody, LabVision/NeoMarkers), mouse monoclonal antibody HPV L1 (clone K1H8, ready to use: Lab Vision, Fremont, CA, USA). The antibody recognized the major L1 including HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, HPV-33, HPV-42, HPV-51, HPV-52, and HPV-58) and monoclonal mouse anti Ki-67 antigen (clone MIB-1, Dako, code: N1633, Denmark; diluted 1:2). The standard Avidin-Biotin Peroxidase Complex **(ABC)** technique was performed using the LabVision Secondary Detection Kit (UltraVision Detection System Anti-polyvalent, HRP). The color was visualized by incubation with chromogen 3, 3' diaminobenzidine for 5 minutes. The slides were then counterstained with Mayer hematoxylin and cover slipped with Permount (StatLab, McKinney, TX). Negative controls were set for each test without the primary antibodies.

Immunohistochemical Evaluation

Results were expressed semi quantitatively. Only cells within the cervical epithelium were counted.

p16 antibody showed reaction in the nucleus, cytoplasm, or both. p16 did not show any reaction with normal epithelial or mesenchymal cells. Interpretation is based on the sum of three parameters; percentage of positive cells, intensity of the reaction, and distribution pattern. Each parameter is graded and a combined score is used to determined positive or negative result based on criteria proposed by Songkhun et al., [16] (Table 1). The percentage of positive cells was scored in the highest expression area (hot spot). The intensity of the reaction is divided into weak, variable (containing weak and strong areas of intensity), and strong. The distribution pattern is interpreted as focal and diffuse. The latter was defined as continuous staining of areas of cells larger than x40 field area.

Positive immunostaining for HPV L1 was identified in the nuclei. Even if only one positive nucleus, it was considered positive for HPV L1 [8,9].

Table 1: P16 Scoring Criteria.

Features		Score
Percentage of positive cells	<5%	0
	5-49%	1
	50-80%	2
	>80%	3
Intensity of the reaction	No reaction	0
	Weak	1
	Variable	2
	Strong	3
Cellular reaction pattern	No reaction	0
	Focal	1
	Diffuse	2
Total score: 0-3 =Negative, 4-8 = Positive		

To determine the grade of Ki-67 expression, nuclei of 200 epithelial cells located across the whole epithelial layer were examined in a high-power field (×400). Ki-67 index was defined as the percentage of Ki-67 positive cells. Grade 1+, 2+, and 3+ was given when the Ki-67 index was below 5%, 5-30%, and greater than 30%, respectively [13,17].

Statistical Analysis

Data analysis was conducted using SPSS version SPSS 13.0 (SPSS Inc., Chicago, IL, USA).The variables of p16; HPV L1 and Ki67 expression are presented. Correlations with different groups were determined. Associations between variables were assessed using nonparametric tests such as the Spearman correlation coefficient by rank test, Kruskal-Wallis test, $\chi 2$ test for independence, and the Mann-Whitney U test. P values of less than .05 were considered statistically significant. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were evaluated for each marker to diagnose CIN2-3 and invasive tumours according to standard protocols [11].

RESULTS

Clinicopatholoical Results

The study included: 10 normal cervical tissues (as a control); 15 cases of CIN 1; 28 cases of CIN 2; 20 cases of CIN 3 and 12 cases of cervical squamous cell carcinoma (SCC). The mean age of each group was as follows: the normal cervical tissue was 37 years, CIN 1 was 29 years, CIN 2 was 38 years, CIN 3 was 49 years and cervical carcinoma was 55 years. The difference among the groups in terms of age was statistically significant (p = 0.04).

P16 Immunostaining in normal cervical tissue, CINs, and cervical carcinoma (Table2)

Positive p16 immunostaining cells appeared as a brown color within the nucleus with or without cytoplasmic staining.

Table 2: Immunoexpression of p16,HPV L1 and Ki67 in cervical squamous intraepithelial lesions.

Studied cases	P16		HPV L1		Ki 67			
	Positive	Negative	Positive	Negative	negative	Grade 1	Grade 2	Grade 3
Control (n=10)	0 (0%)	10 (100%)	0 (0%)	10 (100%)	10 (100%)	0 (0%)	0 (0%)	0 (0%)
CIN 1 (n=15)	4 (27%)	11 (73%)	0 (0%)	15 (100%)	9 (60%)	6 (40%)	0 (0%)	0 (0%)
CIN 2 (n=28)	15 (54%)	13 (46%)	26 (93%)	2 (7%)	0 (0%)	10 (36%)	14 (50%)	4 (14%)
CIN 3 (n=20)	17 (85%)	3 (15%)	8 (40%)	12 (60%)	0 (0%)	0 (0%)	10 (50%)	10 (50%)
Invasive carcinoma (n=12)	12 (100%)	0 (0%)	0 (0%)	12 (100%)	0 (0%)	0 (0%)	0 (0%)	12 (100%)
P value	0.001		0.02		0.003			

Among the normal samples, all cases were negatively stained for p16. The reaction seen was only focal and basal in 4 cases. P16 expression increased during the progression from CIN 1 through CIN 2-3 to cervical carcinoma. Positive staining for p16 was observed in 27 % (4/15) of CIN 1 cases (Figure.1a), 54% (15/28) of CIN 2 cases (Figure.2a), 85% (17/20) of CIN 3 cases (Figure.3a), and 100% (12/12) of SCC cases (Figure.4a). The differences among normal cervical epithelium, CIN 1, CIN 2, CIN 3 and SCC were statistically significant (p = 0.001).

HPV L1 Immunostaining in normal cervical tissue, CINs, and cervical carcinoma (Table2)

Positive immunostaining for HPV L1 was identified in the nuclei of infected cells. HPV L1 capsid protein positivity was observed in the cells of the upper epithelial layers but not in basal and parabasal cells. No positivity was detected in the normal cervical tissue. The detecting rates for HPV L1 was 0 % (0/15) of CIN 1 cases, 93% (26/28) of CIN 2 cases (Figure.2b), 40% (8/20) of CIN 3 cases (Figure.3b), and 0% (0/12) of SCC cases (Figure.4b). It was decreased gradually according to the severity of cervical dysplasia. The HPV L1 capsid protein expression was related significantly to the grade of cervical lesions (p=0.02).

Table 3: Correlation between p16, HPV L1 and Ki67 in cervical squamous intraepithelial lesions.

		Ki67				P value
		Negative (n=9)	Grade 1 (n=16)	Grade 2 (n=24)	Grade 3 (n=26)	
P16	Negative (n=27)	9	16	2	0	0.001
	Positive (n=48)	0	0	22	26	
HPV L1	Negative (n=41)	9	12	8	12	0.05
	Positive (n=34)	0	4	16	14	

Ki 67 Immunostaining in normal cervical tissue, CINs, and cervical carcinoma (Table2)

No positivity was detected in the normal control tissue. On the other hand, Ki-67 expression was detected in all other cases. In CIN 1, 40% (6/15) showed weak Ki-67 expression (Figure.1b), In CIN 2 most of the cases were either grade 2 (Figure.2c) or grade 3 representing 86% (24/28) but 50% (10/20) of patients with CIN 3 had strong Ki67 expression (Figure.3c). Also all the cases of SCC showed strong Ki67 expression (Figure.4c). As the grade of the epithelial lesions was increased, stronger Ki-67 expressions were observed (p=0.003).

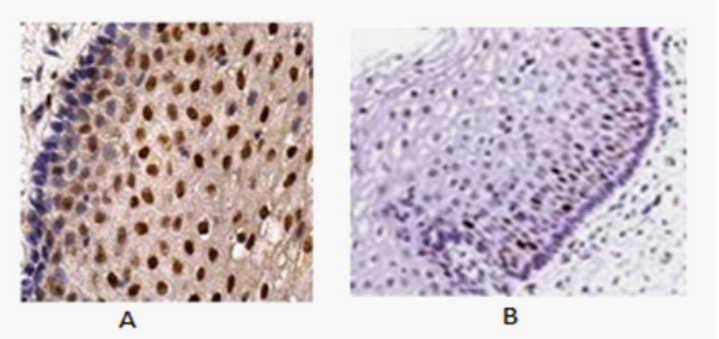


Figure 1: A case of CIN1 showing positive staining for P16 (ax200) and grade 1 ki67 positivity (bx200).

Figure 2: A case of CIN2 showing positive staining for both P16 (ax200) and HPVL1 (bx200) and grade 2 ki67 positivity (cx200).

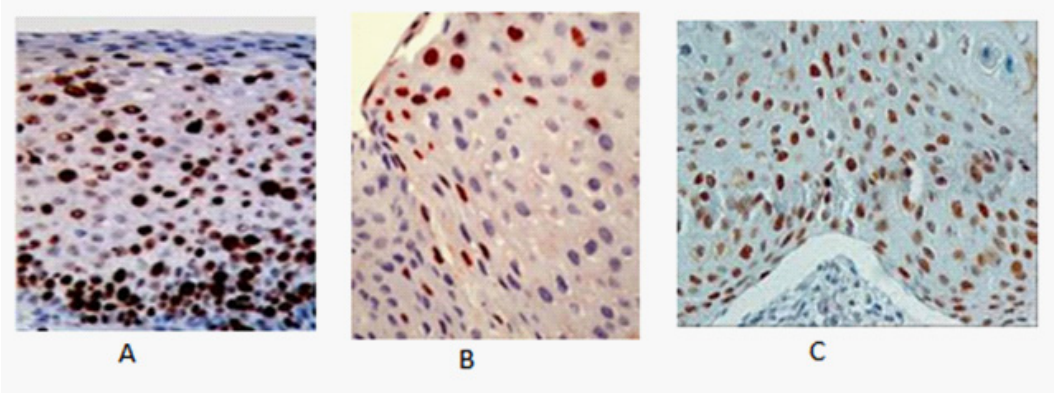


Figure 3: A case of CIN3 showing positive staining for both P16 (ax200) and HPVL1 (bx200) and grade 3 ki67 positivity (cx400).

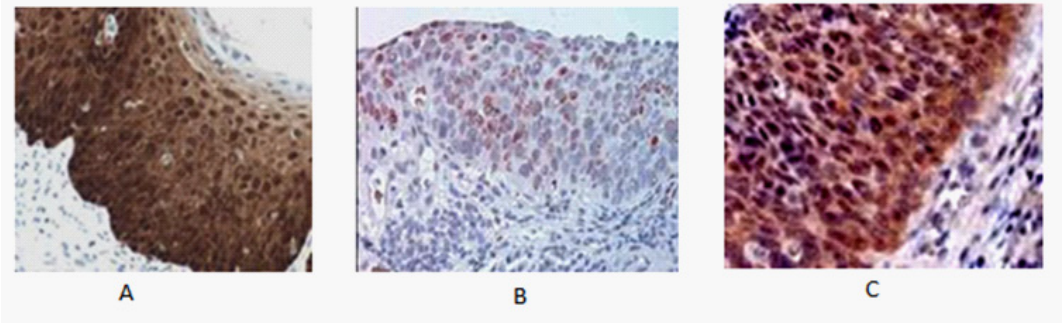
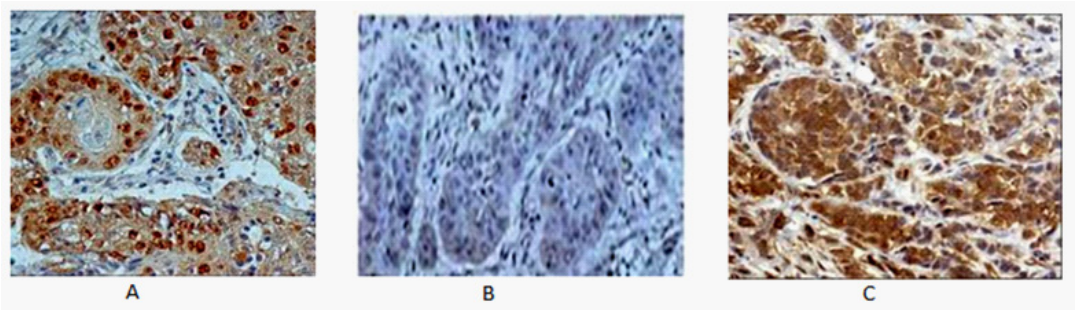


Figure 4: A case of SCC showing positive staining for P16 (ax200), negative for HPVL1 (bx200) and grade 3 ki67 positivity (cx200).



Correlation between p16, HPV L1 and Ki67 in cervical squamous intraepithelial lesions (Table3)

Immunohisto chemical staining results for p16, HPV L1 were correlated with Ki-67. P16 was found to be statistically correlated with Ki67 expression (p=0.001). All negative p16 cases were either negative or weekly expressing (grade 1) Ki67 and all positive P16 cases were either

moderately (grade2) or strongly (grade3) expressing Ki67.On the other hand, HPV L1 positivity decreased with increasing intensity of Ki-67 and this was statistically significant (p=0.05).

Sensitivity, specificity, PPV, NPV and accuracy of the studied markers in diagnosis of cervical squamous intraepithelial lesions (Table4)

- **P16**

Sensitivity of p16 was the best in detecting CIN3 lesions. Specificity of p16 was the best in detecting CIN3, while it was very accurate in detecting CIN2 and CIN3

- **HPV L1**

Sensitivity of HPV L1 was the best in detecting SCC and CIN3 and its specificity was the best in differentiating CIN2 and CIN3, while it was very accurate in differentiating CIN2

- **Ki67**

Sensitivity of Ki67 was the best in differentiating all lesions from each others. The best specificity and highest accuracy were detected in differentiating CIN2 and CIN3.

Table 4: Sensitivity, specificity and accuracy in detecting cervical squamous intraepithelial lesions.

		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy
CIN2,CIN3 vs. CIN1	P 16	67	73	89	41	93
	HPV L1	71	100	100	52	78
	Ki 67	100	60	89	100	90
CIN2 Vs. CIN1	P 16	54	73	79	46	60
	HPV L1	93	100	100	88	95
	Ki 67	100	60	82	100	86
CIN3 Vs. CIN2	P 16	85	46	53	83	63
	HPV L1(-)	60	93	32	20	29
	Ki 67	100	0	42	0	42
SCC Vs. CIN3	P 16	100	15	44	100	47
	HPV L1(-)	100	40	50	100	63
	Ki 67	100	0	50	0	38
SCC Vs. CIN2,CIN3	P 16	100	33	27	100	47
	HPV L1(-)	100	71	26	100	43
	Ki 67	100	0	20	0	20

DISCUSSION

Cervical cancer is a leading malignancy, threatening the health of women, especially in the developing and the underdeveloped countries. It is the second most common malignancy worldwide [9].

There are several reports on the value of p16 in diagnosing CIN [9,18]. In the present study, we found that p16 expression increased from normal tissue (no expression) to invasive cervical cancer (100% expression). It was previously reported that p16 immunostaining in CIN may be a predictor of disease progress that need more intensive follow-up [19,20]. p16 was found to be a useful marker in diagnosis CIN and establishing the risk of CIN 2-3. The sensitivity was the best in detecting CIN3 lesions. Specificity and accuracy were the best in differentiating CIN1 from more aggressive lesions. p16 is useful in distinguishing high-grade CIN from CIN1 but probably not useful for distinguishing CIN1 from non-CIN cases (negative) and this was in agreement with Mary et al., [15], These findings are consistent with previous studies in the potential utility of p16 as a predictive CIN marker [9-11]. The present study observed that there is no differentiation in the distribution of the stain inside the cells among CIN grades, while previous studies reported overexpression of p16 in the cytoplasm in higher grade lesions that may reflect the increased synthesis of p16 [18,21]. The present study found a continuous staining pattern in the nucleus and sometimes in the cytoplasm that extended upward in the cells in proportion to the lesion grade that was consistent with Galgano et al. [22].

The HPV L1 capsid protein is the major target for the cell-mediated immune response to the HPV infections and it is detectable during the productive stage of HPV disease. It is produced abundantly in moderate to severe dysplasia but rarely is observed in nonsuspicious or in carcinomas [9]. This was in accordance with the present study where HPV L1 was negative in CIN 1 cases, increasing in CIN 2 cases, while decreasing in CIN 3 cases, and negative in carcinoma cases. So the expression of HPV L1 capsid protein reduced with the increase of the histological grade of cervical cells and was negatively related to the grade of cervical lesions. HPV L1 capsid protein may serve as markers for the early diagnosis and prediction of cervical lesions. This was in agreeing with Ming et al., [4] and Huang et al., [7]. Sensitivity of HPV L1 was the best in detecting SCC and CIN3. Specificity and accuracy were the best in differentiating CIN2-3. Our results were unlike Mary et al., [15] results who stated that HPV L1 protein detection was neither sensitive nor specific for any class of cervical lesions.

Henrik et al., [23] and Raluca et al., [24] reported that because HPV L1 capsid protein is the major target of cellular immune responses, its loss at early stages of the transformation process may led to ineffective stimulation of immune responses. Therefore, lack of HPV L1 capsid protein may occur to reduce the cellular immune responses, thereby promoting further transformation of epithelial cells. The combination of HPV L1 capsid protein and p16 appears to be useful for an early diagnosis of precancerous lesions, because the HPV L1/p16 expression status may be able

to identify individuals at risk of lesion progression and may also be helpful for subsequent follow-up of patients.

Several molecular markers have been developed to improve the detection of cervical precancerous lesions. Despite a diagnostic value of p16, there are various exceptions that it should be used in combination with other markers in establishing a diagnosis in a routine clinical setting. Nicolas et al., [14] and Sangho et al., [25] stated that Ki67 has been used in combination with p16 because it can be of help for making a differentiation of a high-grade CIN from benign mimickers. In the present study, no Ki67 positivity was detected in the normal control tissue. Ki-67 expression was detected in all other cases. It was observed that it was increasing gradually among CIN grades till reaching 100% in carcinoma cases, indicating that Ki67 expression is a useful adjunct test in the evaluation of low-grade from high grade lesions of the cervix and this was in agree with Alvaro et al., [26] and Fatemeh et al., [13]. Its sensitivity was the best in differentiating various CIN grades while its specificity and accuracy were the best in differentiating CIN2-3. Our results are similar to Mary et al., [15] who stated that Ki-67 staining was equally sensitive but less specific for CIN3 and CIN2 compared to p16.

In the present study, p16 was correlated with Ki67 expression and showed significant difference (p=0.001). Eun et al., [17] found that as the CIN grade increased, the p16 and Ki-67 expressions became stronger. These findings together with the current findings suggest that p16 and ki67 may be involved in the HPV-induced carcinogenesis. Abeer et al., [27] stated that the infection of cervical mucosa by high risk HPV led to deregulation of the cell cycle via altering the expression level of certain genes. These alterations led to acceleration of the cell cycle with increased proliferation rate, as indicated by a high Ki67, and acquisition of more genetic damage.

On the other hand, HPV L1 positivity decreased significantly with increasing intensity of Ki67 (p=0.05). This was in agree with Ming et al., [4] who found that the detection of HPV L1 has been suggested to be useful for predicting progression of CIN and HPV L1 correlate not only with CIN grade but also with disease progression as demonstrated by Ki67 staining.

Finally, Management of CIN remains an important health care problem despite recent advances, including the development of effective prophylactic HPV vaccines. Development of markers that can predict the lesion regression versus persistence/progression would be extremely useful for clinical management. Major capsid protein L1 constitutes the primary structural element of viral capsid. It also represents a major target for the cell-mediated immune response.

It can be concluded that immunohistochemical detection p16 expression can be used as a specific diagnostic marker of cervical dysplasia and cervical cancer, and possibly as a surrogate marker for HPV infection. We recommend using combination of p16, L1 capsid protein and Ki-67 immunostaining as useful objective biomarkers to predict CIN2-3 and this could help in early diagnosis of cervical carcinoma since alterations affecting the expression level of these proteins occur at an early stage of cervical carcinogenesis.

References

1. WB Seung, L Ahwon, K Suyeon, JC Yeong, SL Youn,et al. Immunostaining of p16INK4a/Ki-67 and L1 Capsid Protein on Liquid-based Cytology Specimens Obtained from ASC-H and LSIL-H Cases. Int J Med Sci. 2013; 10 :1602–1607.

2. Nayereh KG, Khadem G. Preventive and Therapeutic Vaccines against Human Papillomaviruses Associated Cervical Cancers. Iran J Basic Med Sci. 2012; 15: 585-601.

3. Omori M, Hashi A, Nakazawa K, Yuminamochi T, Yamane T. Estimation of prognoses for cervical intraepithelial neoplasia 2 by p16INK4a immunoexpression and high-risk HPV in situ hybridization signal types. Am J Clin Pathol. 2007; 128: 208-217.

4. Guo M, Baruch AC, Silva EG, Jan YJ, Lin E. Efficacy of p16 and ProExC immunostaining in the detection of high-grade cervical intraepithelial neoplasia and cervical carcinoma. Am J Clin Pathol. 2011; 135: 212-220.

5. FATavassoli, P Devilee. World Health Organization Classification of Tumor. In: Pathology and Genetics. Tumors of the breast and female genital organs. Lyon: IARC Press. 2003; 260–289.

6. Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D. The 2001 Bethesda System: terminology for reporting results of cervical cytology. JAMA. 2002; 287: 2114-2119.

7. Bin H, Ruifang W, Ruizhen L, Yiheng L, Zhihong L. Detention of HPV L1 Capsid Protein and hTERC Gene in Screening of Cervical Cancer. Iran J Basic Med Sci. 2013; 16: 797-802.

8. A Yemelyanova, PE Gravitt, BM Ronnett, AF Rositch, A Ogurtsova, et al. Immunohistochemical detection of Human Papillomavirus capsid proteins L1 and L2 in squamous intraepithelial lesions: Potential utility in diagnosis and management. Mod Pathol. 2013; 26: 268–274.

9. Haltas H, Bayrak R, Yenidunya S, Yildirim U. The immunohistochemical detection of p16 And HPV l1 capsid protein on cell block Sections from residual papspin liquid-based Gynecology cytology specimens as a diagnostic and prognostic tool. European review for medical and pharmacological sciences. 2012; 16: 1588-1595.

10. TR Maria, HT José, T Maricy, GP Silvana, HC Hélio, et al. Characterization of p16 and E6 HPV-related proteins in uterine cervix high-grade lesions of patients treated by conization with large loop excision. Oncol Lett. 2013; 6: 63–68.

11. FN Alcina, EG Jonathan, RL José, BC Cynthia, MA Sergio, et al. An evaluation of p16INK4a expression in cervical intraepithelial neoplasia specimens, including women with HIV-1. Mem Inst Oswaldo Cruz, Rio de Janeiro. 2012; 107: 571-577.

12. Denton KJ, Bergeron C, Klement P, Trunk MJ, Keller T. The sensitivity and specificity of p16(INK4a) cytology vs HPV testing for detecting high-grade cervical disease in the triage of ASC-US and LSIL pap cytology results. Am J Clin Pathol. 2010; 134: 12-21.

13. Sari Aslani F, Safaei A, Pourjabali M, Momtahan M. Evaluation of Ki67, p16 and CK17 Markers in Differentiating Cervical Intraepithelial Neoplasia and Benign Lesions. Iran J Med Sci. 2013; 38: 15-21.

14. Wentzensen N, Schwartz L, Zuna RE, Smith K, Mathews C. Performance of p16/Ki-67 immunostaining to detect cervical cancer precursors in a colposcopy referral population. Clin Cancer Res. 2012; 18: 4154-4162.

15. Galgano MT, Castle PE, Atkins KA, Brix WK, Nassau SR. Using biomarkers as objective standards in the diagnosis of cervical biopsies. Am J Surg Pathol. 2010; 34: 1077-1087.

16. V Songkhun, K Anant, S Cheepsumon, T Kobkul, K Pilaiwan, et al. Interobserver Reproducibility in Determining p16 Overexpression in Cervical Lesions: Use of a Combined Scoring Method. Asian Pacific Journal of Cancer Prevention. 2008; 9: 653-657.

17. Nam EJ, Kim JW, Hong JW, Jang HS, Lee SY. Expression of the p16 and Ki-67 in relation to the grade of cervical intraepithelial neoplasia and high-risk human papillomavirus infection. J Gynecol Oncol. 2008; 19: 162-168.

18. Queiroz C, Silva TC, Alves VA, Villa LL, Costa MC. P16(INK4a) expression as a potential prognostic marker in cervical pre-neoplastic and neoplastic lesions. Pathol Res Pract. 2006; 202: 77-83.

19. Izadi-Mood N, Asadi K, Shojaei H, Sarmadi S, Ahmadi SA. Potential diagnostic value of P16 expression in premalignant and malignant cervical lesions. J Res Med Sci. 2012; 17: 428-433.

20. MA Yuan-ying, CH Xiao-dong, ZH Cai-yun, QI Li-qian, CH Xiao-duan, et al. Value of p16 expression in the triage of liquid-based cervical cytology with atypical squamous cells of undetermined significance and low-grade squamous intraepithelial lesions. Chinese Medical Journal. 2011; 124: 2443-2447.

21. Lesnikova I, Lidang M, Hamilton-Dutoit S, Koch J. p16 as a diagnostic marker of cervical neoplasia: a tissue microarray study of 796 archival specimens. Diagn Pathol. 2009; 4: 22.

22. Galgano MT, Castle PE, Atkins KA, Brix WK, Nassau SR. Using biomarkers as objective standards in the diagnosis of cervical biopsies. Am J Surg Pathol. 2010; 34: 1077-1087.

23. Griesser H, Sander H, Walczak C, Hilfrich RA. HPV vaccine protein L1 predicts disease outcome of high-risk HPV+ early squamous dysplastic lesions. Am J Clin Pathol. 2009; 132: 840-845.

24. B Raluca, S Nicoleta, EG Simona, G Adriana, GV Laura, et al. Immunohistochemical assessment of p16, COX-2 and EGFR in HPV-positive cervical squamous intraepithelial lesions. Rom J Morphol Embryol. 2011; 52: 1187–1194.

25. Lee S, Kim H, Kim H, Kim C, Kim I. The Utility of p16INK4a and Ki-67 as a Conjunctive Tool in Uterine Cervical Lesions. Korean J Pathol. 2012; 46: 253-260.

26. Pinto AP, Schlecht NF, Woo TY, Crum CP, Cibas ES. Biomarker (ProEx C, p16(INK4A), and MiB-1) distinction of high-grade squamous intraepithelial lesion from its mimics. Mod Pathol. 2008; 21: 1067-1074.

27. Bahnassy AA, Zekri AR, Saleh M, Lotayef M, Moneir M. The possible role of cell cycle regulators in multistep process of HPV-associated cervical carcinoma. BMC Clin Pathol. 2007; 7: 4.