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RESEARCH ARTICLE

p16INKa and Ki67 expression in different grades of cervical intraepithelial neoplasm in relation to HPV genotypes

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ABSTRACT

Background: Early diagnosis of cervical intraepithelial neoplasia and prevention of its malignant progression is very important. The interaction between human papillomavirus (HPV) oncogenic proteins and the cellular regulatory proteins leads to the upregulation of p16INK4a. Overexpression of p16INK4a is a surrogate biomarker of HPV infection leading to premalignant or malignant cervical lesions. **Aim:** In this study, we evaluated the expression of p16INK4a/Ki67 in correlation with HPV genotypes to find out their correlation with dysplasia grading. Patients and **Methods:** Pap cytology and HPV genotyping were performed from 101 women referred to colposcopy. Biopsy specimens were stained with haematoxylin-eosin, then sections were used for subsequent p16INK4a and Ki67 immunohistochemical analysis. **Results:** There was a significant correlation between the staining score of p16INK4a and Ki67 and increase the severity of the cervical lesion. A significant positive correlation was found between HPV type and cervical lesion severity. A significant correlation was found between HPV genotypes and the expression of either p16INK4a or Ki67. **Conclusion:** There is a strong association between the degree of dysplasia and combined p16INK4a/Ki67 immunoreactivity which could be explained by cervical malignant transformation associated with high-risk HPV infections.

Keywords: Cervical carcinoma, Cervical intraepithelial neoplasm, HPV genotypes, Ki67, p16INK4a

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INTRODUCTION

Cervical carcinoma is the third (Arbyn, 2011) or the fourth (Arbyn, 2020; Han, 2020) most common malignancy in women worldwide accounting for about 7.9% of all cancers. It is considered the most common cause of death among women in developing countries (Arbyn, 2011). Cervical carcinoma is a preventable disease that can be controlled by early detection and management (McLaughlin-Drubin, 2013). Human papillomavirus (HPV) infection of the cervix, mainly high-risk (HR) types, is a major cause of cervical dysplasia and carcinoma (Kalyani, 2015). The progression of cervical squamous cells carcinoma (SCC) from premalignant HPV-driven lesions does not occur in all patients. Although a significant portion of cervical intraepithelial neoplasm (CIN) II cases progress to CIN III, a regression can also happen. No clear definitive way to determine which CIN II lesions will progress, and which will regress (Darragh, 2012). Therefore, it is important to find biomarkers that can predict the clinical outcomes and guide the management of CIN II (Gustinucci, 2012; Sari Aslani, 2013). According to the Lower Anogenital Squamous Terminology (LAST) standardization project for HPV-associated, p16INK4a immunohistochemistry should be considered when a cervical biopsy is histopathologically diagnosed as CIN II to determine if the lesion should be considered as a low or high-grade lesion (Darragh, 2012). The HPV oncoproteins that are most linked to the

development of cervical neoplasia are E6 and E7 (Klaes, 2001; Nam, 2008 & Arbyn, 2020). These oncoproteins bind to regulatory tumour suppressor proteins p53 and Rb causing their degradation and functional inactivation, respectively. The binding of the E7 oncoprotein to Rb releases the E2F from its complex with hypophosphorylated Rb and causes cell cycle progression. The functional inactivation of Rb thus causes overexpression of the CDK inhibitor p16INK4a thereby regulating CDK4 and CKD6 to control the cell proliferation (Agoff, 2003). Consequently, HPV-mediated cervical carcinoma and dysplasia overexpress p16INK4a. Ki67, a reliable marker of DNA replication, and cell proliferation are expressed in cervical neoplasia and dysplasia (Izadi-Mood, 2012). It is very helpful and extremely important to find a diagnostic method that can elucidate the extent of preneoplastic cervical lesions. Thus, we postulated that combined p16INK4 and Ki67 expression may have a predictive value in patients with cervical biopsies diagnosed as high grade. Therefore, the management strategy will be changed for patient benefit.

MATERIAL AND METHODS

A retrospective study was done; specimens were collected from women who underwent cervical biopsy from January 2018 to December 2020 in private hospitals in Doha Qatar. The present study was in accordance with the ethical standards of the responsible committee on national human experimentation and according to the Helsinki Declaration of 1975, revised in 2008. A total of 101 biopsies were collected from women who have a history of abnormal Pap smear. First cervical cells were obtained from the internal genitalia with a Cytobrush (SurePath) for HPV DNA extraction, then biopsy specimens were fixed in formalin, embedded in paraffin, and stained with haematoxylin-eosin. Sections were used for subsequent p16INK4a and Ki-67 immunohistochemical analysis.

HPV DNA detection

The HPV hybrid capture II® kit (Digene/Abbott, Clopper Road, Gaithersburg, Maryland, USA) was used to detect different types of HPV. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68 were considered as the HR- HPV, and HPV 6, 11, 34, 40, 42, 43, 44, 70 were regarded as the low risk - HPV (LR- HPV).

Immunohistochemistry staining and scoring

All tissue blocks were cut to provide sections of 3 µm, then immunostaining was performed with mouse monoclonal antibodies against p16INK4a antigen (Roche, CINtec, Clone E6H4)

and Ki67 antigen (DAKO, Clone MIB-1), using the automated IHC Ventana staining machine (Roche, Basel, Switzerland).

Scoring of p16INK4a and Ki67

Diffuse or ‘block’ cytoplasm or nucleus staining in the epithelial cells for p16INK4a was considered positive. A four-semiquantitative method was used to determine the score of p16IK4a expression, according to the staining intensity and area extend of positively stained tumour cells: Score 0 (negative) is defined as either no p16INK4a positivity or focally scattered positive cells or small cell clusters (i.e., patchy staining). Score 1 (weak) is defined as low intensity, but diffuse positivity restricted to the lower one-third part of the epithelium. Score 2 (intermediate) is defined as continuous positivity in the lower two-thirds of the epithelium. Score 3 (strong) is defined as positive cells involving the diffuse full thickness of the epithelium. It is considered important to distinguish positive diffuse or ‘block’ p16INK4a staining from patchy or background staining.

Nuclear Ki67 staining in epithelial cells was scored as positive. Score 0 (negative) is a normal staining pattern (staining of nuclei in the basal layer). Score 1 (weak) is defined as positive nuclei predominantly found in the lower one-third of the epithelium. Score 2 (intermediate) is defined as positive nuclei predominantly found in the lower two-thirds of the epithelium. Score 3 (strong) is defined as positive nuclei in more than two-thirds of the epithelium. The presence of scattered positive individual cells in the upper two-thirds of the epithelium in a predominant staining pattern in the lower one-third is considered as score 1 (weak). Also, a few scattered positive cells found in the upper one-third layer of the epithelium in a predominant pattern with two-thirds involvement of the epithelium is considered as score 2 (intermediate). Areas, where dermal papillae

narrow down the width of the epithelium, cannot be scored reliably and are therefore not considered.

Statistical analysis

Number and percentage were used to describe categorical variables as mean ± standard variation, while the range was used for continuous variables. Data were analysed by SPSS 26 software (IBM Corp., Armonk, NY, USA), using a descriptive statistic. The expression of p16INK4a and Ki67 staining in association with the clinical data and HPV types were compared using the Chi-square test. A p-value < 0.05 was considered significant.

RESULTS

Clinicopathological Characteristics

As depicted in table 1, a total of 101 patients were involved in the work with a mean age and stander deviation (SD) of 31.50 **±** 6.103, most of the cases were found in the age group 20 to 40 years (84 cases, 83.2%). Most of the cases (31 cases, 30.7%) had a history of ASCUS while only one case (1 case, 1%) had a history of leukoplakia. A total of 81 cases were subjected to HPV testing, of which only 10 cases (9.9%) were negative, while the remaining cases were positive with nearly equal numbers of either LR-HPV (36 cases, 35.6 %) or HR-HPV cases (35 cases, 34.7 %). Cervicitis was the diagnosis in 34 cases (33.7%), while CIN I (fig. 1A), CIN II (fig. 2A), and CIN III (fig. 3A) were found in (30 cases, 29.7 %), (21 cases 20.8%) and (16 cases, 15.8 %) respectively. The correlation between histopathology results with either clinical history or age groups was insignificant (P = 0.242 and 0.303 respectively).

Correlation between p16INK4a expression and the severity of the cervical lesion.

A significant correlation was detected between the expression of p16INK4a with increased severity of the cervical lesion (P = 0.000), as showed in table 2. Cases of cervicitis were negative for p16INK4a except for 5 cases (14.7 %) that showed weak staining. In contrast, the majority of CIN II cases showed intermediate staining intensity (11 cases, 52.4%) (fig. 2B) while most of CIN III cases showed strong staining intensity for p16INK4a (12 cases, 75%) (fig. 3B). Regarding CIN I cases, they showed either negative or weak staining (fig. 1B) but never showed a strong intensity.

Correlation between Ki67 expression and the severity of the cervical lesion.

As illustrated in table 3, a significant correlation was found between Ki67 expression and increase in the severity of the cervical lesion (P = 0.001). Cases of cervicitis are almost negative for Ki67 except for 2 cases (5.9 %) that showed weak staining. In contrast, most of CIN II showed intermediate staining (11 cases, 52.4%) (fig. 2C), while the majority of CIN III cases, showed strong staining (11 cases, 68.8%) (fig. 3C). CIN III did not show weak staining at all, while only 3 cases (14.3 %) of CIN II cases showed weak staining. Regarding CIN I cases, they were either negative or show weak to intermediate staining for Ki67(fig. 1C) but never showed a strong staining score.

Correlation between combined staining patterns for both p16INK4a and Ki67.

Cases with intermediate or strong intensity for p16INK4a never showed negativity or weak staining for Ki67 as illustrated in table 4. Cases negative for p16INK4a never showed intermediate or strong staining for Ki67, however, it showed a weak staining pattern for Ki67 (3 cases, 8.6%). Cases with weak staining for p16INK4a never showed strong staining for Ki67, however, it showed mostly weak staining (22 cases, 71.0%) and to a lesser extent, intermediate staining (5 cases, 16.1%), and this was significantly correlated (p-value = 0.000).

The correlation between HPV genotypes and the severity of the cervical lesions.

As shown in table 5, patients with a history of cervicitis are either negative for HPV or positive for LR-HPV (9 cases, 39.1%) or HR-HPV (6 cases, 26.1 %), while patients with a history of CIN I are mostly showing positivity for LR-HPV (21 cases, 80.8%). Regarding patients with a history of CIN II and CIN III, they are mostly positive for HR-HPV (15 cases, 78.9% and 11 cases, 84.6% respectively) and this was significantly correlated (p-value = 0.001). The correlation between HPV with either clinical history or age groups was insignificant (p-value 0.185 and 0.115 respectively).

Correlation between HPV DNA genotype and p16INK4a and Ki67 staining score.

As observed in table 6, a significant correlation was found between HPV genotype and the expression of either p16INK4a or Ki67 (p-value = 0.000 for each). HPV negative cases did not show intermediate or strong staining scores for either p16INK4a or Ki67 but showed weak staining, for p16INK4a (4 cases, 40.0%) and Ki67 (2 cases, 20.0%). Most of HR-HPV positive cases showed a strong staining score for both p16INK4a and Ki67 with only 14.3% (5 cases) were negative for both. LH-HPV positive cases were mostly either negative for p16INK4a (12 cases, 33.3%) or Ki67 (11 cases, 30.6%) or showed a weak staining pattern for p16INK4a (16 cases, 44.4%) and Ki67 (13 cases, 36.1%).

DISCUSSION

The only malignant tumour in the world with well-known causes and preventable measures is cervical cancer. The occurrence of cervical cancer is attributed to persistent infection with HPV (Bulk, 2006; Wang, 2018). However, CIN I caused by general HPV infection usually resolves spontaneously with no additional treatment. However, some CIN I will transform into CIN II or CIN III, which will progress to SCC (Sano, 2002). Screening for HPV risk types alone is not a sufficient factor for cervical cancer detection. It is highly sensitive for the detection of precancerous lesions but cannot differentiate the precancerous cases from merely HPV-infected individuals (Schiffman, 2010). We investigated the correlation between p16INK4a/Ki67 co-testing independently and in combination with HPV DNA genotypes to detect cervical precancerous lesions. In the current study, patients were stratified into three age groups, the most frequent one was 20 to 40 years (83.2%) and no significant correlation was detected between age groups and HPV genotypes or the severity of the cervical lesion (P = 0.115 and 0.303 respectively). According to HPV DNA genotype patients were stratified into three groups including HPV negative cases (9.9%), LR- HPV (35.6%), and HR-HPV (34.7%). The most frequent clinical presentation was ASCUS (30.7%). Patient biopsy results showing cervicitis in (33.7%) of cases, followed by CIN I (29.7%), CIN II (20.8%), and CIN III (15.8%).

In the present study, a significant positive correlation (p-value = 0.000) was detected between p16INK4a expression and the severity of cervical lesion; the strongest staining was most frequently found in CIN III (75%), the intermediate staining was mostly detected in CIN II (52.4%), while cases of CIN I or cervicitis never showed p16INK4a strong staining score or showed only weak staining if any. This result was in accordance with previous studies that reported that diffuse intense p16INK4a expression was found in 80% to 100% in invasive carcinoma, 45% to 100% in CIN2/3, and 0% to 15% in non-dysplasia (Volgareva, 2004; Wang, 2004; Sari Aslani, 2013; Kanthiya, 2016). The variation of expression rates may be attributable to the criteria defining positive expression. In CIN III, intensive staining of p16INK4a antigen can be seen both nuclear and cytoplasmic. The diffusely positive p16INK4a antigen staining is a hallmark distinguishing carcinoma in situ from microinvasive carcinoma (Gatta, 2011; Nuovo, 2016). Also, Klaes (2002) reported diffuse and strong expression of p16INK4a in high-grade CIN, on the other side inflammatory and metaplastic cervical lesions show negative staining Differently, in infections caused by low-risk HPV types, the p16INK4a immunostaining pattern displayed weak and focal staining, located in nuclei and cytoplasm of the intermediate and superficial layers only and if CIN I showed diffuse p16INK4a immunostaining it had higher chances of progression to CIN 2-3 (Krishnappa, 2014). One study which found a higher rate of p16INK4a expression in CIN, and invasive cancer also found a higher rate of expression in non-dysplasia (32%) than other studies (0% to 15%) (Wang, 2004), which may attribute to the criteria used in that study or it may attribute to HPV infection genotypes.

This study revealed a significant correlation between ki67 expression and severity of the cervical lesion (P = 0.001). CIN II and CIN III showed strong positive staining for Ki67 (33.3% and 68.8% respectively) while cases of CIN I and cervicitis show only 5.9% and 0.0% respectively. These results agree with what was reported earlier that Ki67 expression was 90% to100% in invasive carcinoma, 20% to 70% in CIN2/3, 70% to 90% in CIN1, and 0% to 20% in non-dysplasia

Table 1. Clinicopathological Characteristics of the study groups

Clinicopathological characteristics of the study groups (Total No. 101)		No	%
Age mean ± SD (31.50 ± 6.103)			
Age groups	1-20 years	7	6.9
	20-40 years	84	83.2
	41-60years	10	9.9
History	Unhealthy cervix	21	20.8
	Leukoplakia	1	1.0
	ASCUS	31	30.7
	LSIL	28	27.7
	ASC-H	8	7.9
	HSIL	12	11.9
HPV (Total No. 81)	HPV-ve	10	9.9
	LR-HPV	36	35.6
	HR-HPV	35	34.7
Histopathology results	Cervicitis	34	33.7
	CIN I	30	29.7
	CIN II	21	20.8
	CINIII	16	15.8

ASCUS atypical squamous cells of undetermined significance, LSIL low grade squamous intraepithelial lesion, ASC-H atypical squamous cells cannot exclude high-grade lesion, HGIL high grade squamous intraepithelial lesion.

Table 2. Correlation between p16INK4a expression and the severity of the cervical lesion.

Histopathology results	p16INK4a				Total No. 101	p-value
	-	+	++	+++		
Cervicitis	29 (85.3%)	5 (14.7%)	0 (0.0%)	0 (0.0%)	34	0.000*
CIN I	6 (20.0%)	22 (73.3%)	2 (6.7%)	0 (0.0%)	30	
CIN II	0 (0%)	4 (19.0%)	11(52.4%)	6 (28.6%)	21	
CINIII	0 (0%)	0 (0.0%)	4 (25%)	12 (75%)	16	

* p-value < 0.05 is considered statistically significant according to the Chi-Square test.

Table 3. Correlation between Ki67 expression and the severity of the cervical lesion.

Histopathology results	Ki67				Total No. 101	p-value
	-	+	++	+++		
Cervicitis	32 (94.1%)	2 (5.9%)	0 (0.0%)	0 (0.0%)	34	0.001*
CIN I	4 (13.3%)	20 (66.7%)	6 (20.0%)	0 (0.0%)	30	
CIN II	0 (0%)	3 (14.3%)	11(52.4%)	7 (33.3%)	21	
CIN III	0 (0%)	0 (0.0%)	5 (31.3%)	11 (68.8%)	16	

* p-value < 0.05 is considered statistically significant according to the Chi-Square test.

Table 4. Correlation between combined staining patterns of both p16INK4a and Ki67.

p16INK4a	Ki67				Total No	p-value
	-	+	++	+++		
-	32 (91.4%)	3 (8.6%)	0 (0.0%)	0 (0.0%)	36	0.000*
+	4 (12.9%)	22 (71.0%)	5 (16.1%)	0 (0.0%)	31	
++	0 (0.0%)	0 (0.0%)	14 (82.4%)	3 (17.6%)	17	
+++	0 (0.0%)	0 (0.0%)	3 (16.7%)	15 (83.3%)	18	

* p-value < 0.05 is considered statistically significant according to the Chi-Square test.

Table 5. The correlation between HPV genotypes and the severity of the cervical lesions.

Histopathology results	HPV			Total No. 81	p-value
	HPV-ve	LR-HPV	HR-HPV		
Cervicitis	8 (34.8%)	9 (39.1%)	6 (26.1%)	23	0.001*
CIN I	2 (7.7%)	21 (80.8%)	3 (11.5%)	26	
CIN II	0 (0.0%)	4 (21.1%)	15 (78.9%)	19	
CIN III	0 (0.0%)	2 (15.4%)	11 (84.6%)	13	

* p-value < 0.05 is considered statistically significant according to the Chi-Square test.

Table 6. Correlation between HPV DNA genotype and p16INK4a and Ki67 intensity scores.

HPV (Total 81)	p16INK4a				Ki67			
	-	+	++	+++	-	++	++	+++
HPV-ve (10 cases)	6(60.0%)	4(40.0%)	0(0.0%)	0 (0.0%)	8(80.0%)	2(20.0%)	0(0.0%)	0(0.0%)
LR-HPV (35 cases)	12(33.3%)	16 (44.4%)	6(16.7%)	2 (5.6%)	11(30.6%	13(36.1%)	8(22.2%)	4(11.1%)
HR-HPV (36 cases)	5(14.3%)	6(17.1%)	10(28.6%)	14(40%)	5(14.3%)	6(17.1%)	12(34.3%)	12(34.3%)
P value	0.000*				0.000*			

* p value < 0.05 is considered statistically significant according to Chi-Square test.

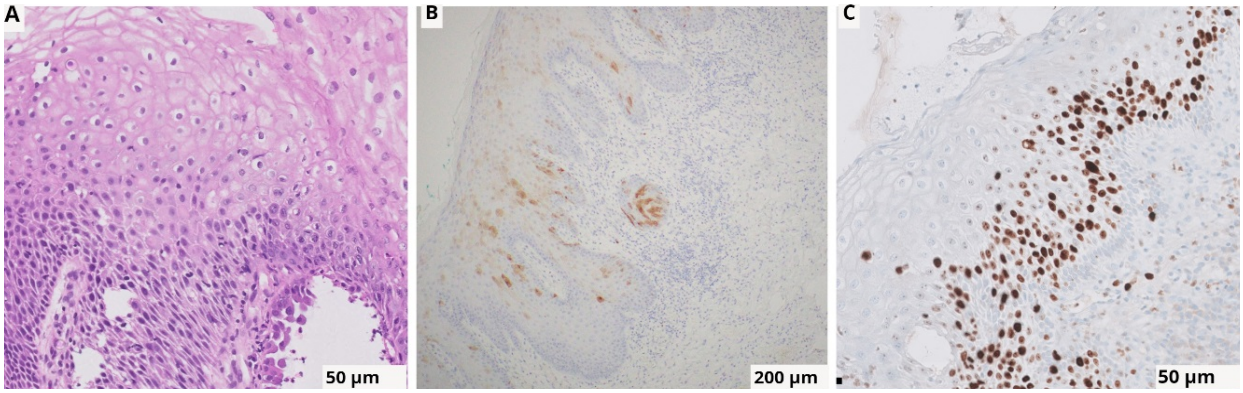

Figure 1. Cervical intraepithelial neoplasm grade I (H & E staining X50) (A). Scattered clusters of p16INK4a positive cells score 1 (p16INK4a IHC, X200) (B). Ki67 positive nuclear staining predominantly found in the lower one-third of the epithelium, score 1 (Ki67 IHC, X50) (C).

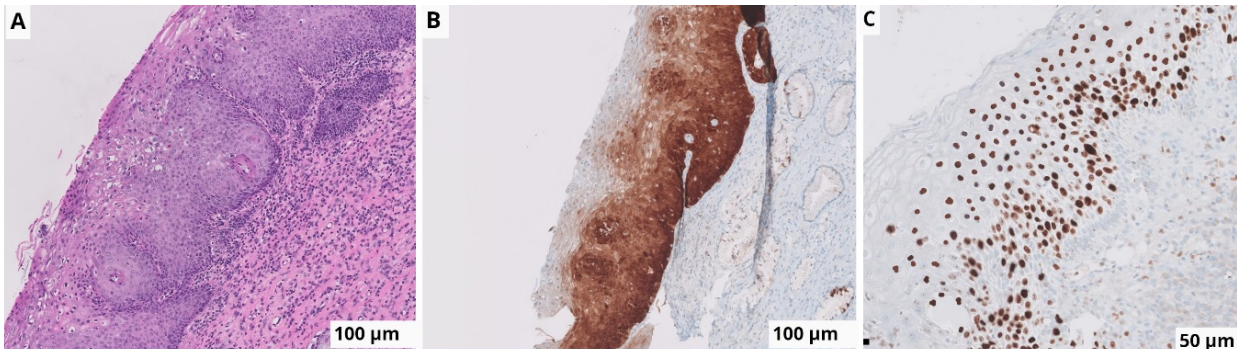

Figure 2. Cervical intraepithelial neoplasm grade II (H & E staining X100) (A). Continuous positive cytoplasmic staining for p16INK4a in the lower two-thirds of the epithelium, score 2 (p16INK4a IHC, X100) (B). Ki67 positive nuclear staining predominantly found in the lower two-thirds of the epithelium, score 2 (Ki67 IHC, X50) (C).

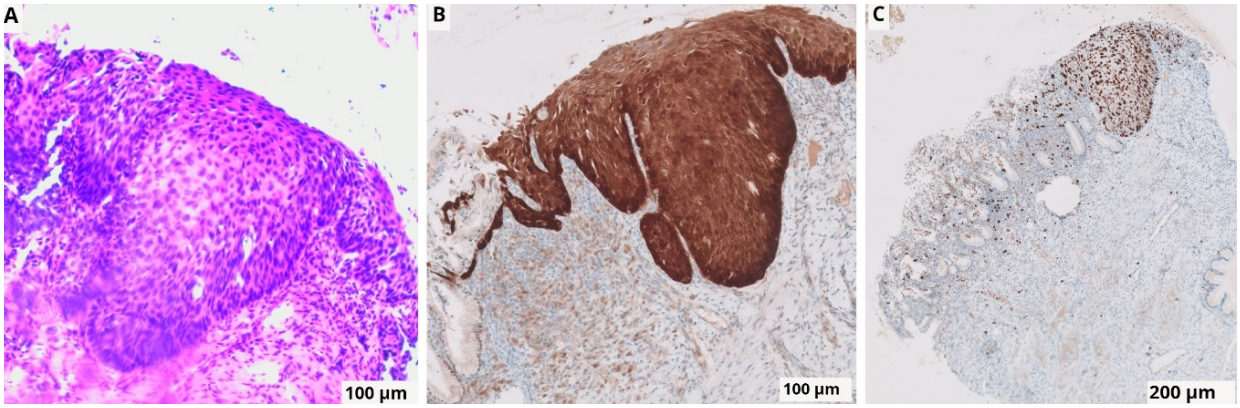

Figure 3. Cervical intraepithelial neoplasm grade III (H & E staining, X100) (A). Strong continuous positive cytoplasmic staining for p16INK4a in the full epithelium thickness, score 3 (p16INK4a IHC, X100) (B). Ki67positive nuclear staining in the full epithelium thickness, score 3 (Ki67 IHC, X200) (C).

(Walts, 2009; Cavalcante, 2012; Jackson, 2012). Immunopositivity for Ki67 linearly increases as the CIN grade is higher (Nam, 2008; Kim, 2011; Bleotu, 2009). Kruse (2001) found that Ki67 to be useful to distinguish between different grades of cervical intraepithelial neoplastic lesions, as a potential method of quality control and a possible indicator of progression in low-grade lesions. Ki67 does not aid differentiation between reactive changes and high-grade dysplasia. Furthermore, quantitative scoring can be complicated by proportional differences in glands seen in the field of the microscope (Samarawardana, 2011). A significant positive correlation was found between combined p16INK4a and Ki67 expression in this study (*P* = 0.000). The expression levels of p16INK4a and Ki67 gradually increase with the development of cervical intraepithelial lesions and cervical cancer (Kimura, 2006; Carreras, 2007; Bleotu, 2009). Moreover, p16INK4a/Ki67 dual staining has been used for cervical cancer screening (Clarke, 2019; Wentzensen, 2019).

The current study revealed that most patients with cervicitis are either negative for HPV or positive for LR-HPV (34.1% and 39.1 % respectively), while patients with a history of CIN I are mostly showing positivity for LR-HPV (80.8%) and patients with a history of CIN II and CIN III are mostly positive for HR-HPV (78.9%, and 84.6% respectively) and this was statistically significant (p-value = 0.001). These results are like what is reported by Nam (2008) who demonstrated that in low-grade CIN, the HPV test was negative in 54.5% of patients. HPV positivity rates increase directly with the cervical lesion severity (Chan, 2012; Ding, 2014; Liu, 2014).

Regarding the correlation between p16INK4a and Ki67 with HPV genotype, it was found that HPV negative cases never showed intermediate or strong staining score for either p16INK4a or Ki67 and most of HR-HPV positive cases showed strong positive staining for both p16INK4a and Ki67and this was statistically significant (*P* = 0.000 for each). This may be explained by a study done by Bosch *et al*. (who found that the p16INK4a overexpression is rare in patients infected with a low-risk HPV because E7 of low-risk HPV has a lower affinity to pRb than that of high-risk HPV (Bosch, 1995). Klaes (2002) and Agoff (2003) found that overexpression of p16INK4a has a close association with high-risk HPV infection and correlates with the degree of cervical neoplasia. p16INK4a and Ki67 co-expression is present in almost all high-grade squamous and glandular lesions and rarely in benign conditions and that the combination of markers is more sensitive and specific than either of them used in isolation (Nam, 2008; Samarawardana, 2011). Keating (2001) proved a strong relationship between Ki67, cyclin E, and p16INK4a in the recognition of HPV-associated precursors as well as in distinguishing normal squamous mucosa from precancerous lesions and, found that with the increase of p16INK4a and Ki67 expression levels, the number of HR-HPV copies increase. The expression of p16INK4a and Ki67 were positively associated with the HR-HPV infection status. Therefore, upregulation of p16INK4a and Ki67 indicating infection with HR-HPV (Nam, 2008; Arbyn, 2020).

In conclusion, there is a strong association between the degree of dysplasia and combined p16INK4a/Ki67 immunoreactivity that could be explained by cervical malignant transformation associated with high-risk HPV infections. The significant differences in these markers' expression may be useful in equivocal histologic features among the cervical intraepithelial lesions. Also, the use of these markers together with HPV testing may help in predicting the progression of lesions that seems to be low grade.

AUTHORS' CONTRIBUTIONS

Design of the work: Amany, Delaram and Hoda. Interpretation of immunohistochemistry results: Delaram and Amany. Analysis, and interpretation of data: Amany and Hoda. Drafting the work: Delaram, Amany and Hoda. Revision: Hoda and Amany. Final approval: Amany, Hoda and Delaram.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTION

Not applicable.

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