Asia-Pacific Journal of Molecular Medicine 2011, 1:6

Expression of p16 and pKi-67 in Cervical Preneoplasia and Neoplasia

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Received on 23/11/2010 / Accepted on 13/06/2011

ABSTRACT

This study was conducted to investigate the expression of p16 and pKi-67 in normal, preneoplasia and neoplasia lesions of the uterine cervix. One hundred and thirty one cervical specimens, consisting of normal cervix (n = 43), cervical intraepithelial neoplasia (CIN) lesions (n = 40) [16 LSIL: CIN 1 and 24 HSIL: 9 CIN 2 and 15 CIN 3] and cervical squamous cell carcinomas (n = 48) [16 SCC I, 17 SCC II, 7 SCC III and 8 SCC IV] were examined immunohistochemically in paraffin sections. All samples of the normal cervix were negative for p16. Immunoreactivity of p16 was observed in 4/16 LSIL, 12/24 HSIL and 30/48 SCC. In all p16-positive samples, both nuclear and cytoplasmic staining were observed. High expression of p16 (> 50 % of cell stained) was found in HSIL and SCC. Ki-67 index was significantly ($p < 0.05$) higher in high-grade squamous intraepithelial lesions (HSIL: CIN 2 & 3) and SCC lesions when compared to normal cervices. The expression of p16 and Ki-67 proliferation profile (< or > 30 % stained cells) were significantly associated with the grade of lesions ($\chi^2 = 6.832$, $p = 0.033$ and $\chi^2 = 10.952$; $p = 0.012$ respectively). There was no significant relationship demonstrated between p16 positivity and Ki-67 proliferation profile ($\chi^2 = 0.292$; $p = 0.589$). Our results indicated that p16 protein may be involved in the carcinogenesis of cervical cancer. However, overexpression of p16 seemed to have no effect on cell proliferation. The expression of p16 and pKi-67 may be useful in cases where it is difficult to make a diagnosis by histology.

INTRODUCTION

Eukaryotic cell cycle progression is controlled through a complex mechanism involving the coordinated expression and post-translational modification (e.g. phosphorylation) of cell cycle regulating proteins. Among these proteins, the cyclin-dependent kinase (CDK) inhibitor p16 plays a crucial role in retinoblastoma (pRb)-mediated control of the G1-S-phase transition of the cell cycle [1].

p16 is a tumor suppressor protein that inhibits CDK4 and CDK6, which phosphorylates pRb and subsequently releasing the transcription factor E2F. A reciprocal relation between p16 and pRb expression has been observed, suggesting the presence of a negative feedback loop allowing pRb to limit the concentration of p16 [2]. However, the regulatory pathway is disrupted by the human papilloma virus (HPV) oncoprotein E7, which is known to bind and inactivate pRb. E2F is subsequently released from pRb-E2F complex hence allowing the cell to enter the S phase [2]. E2F accumulation may also lead to induction of p16 [2]. Many studies have proposed p16 as a biomarker and an additional screening tool for cervical lesions [3-5].

pKi-67 is expressed during all phases of cellular cycle (G1, S, G2 and M of proliferating cells), but is absent in quiescent cells (G0). It is therefore a reliable biomarker of cellular proliferation which can be determined immunohistochemically [6]. The study aim was to identify biomarkers of cellular proliferation in cervical lesions by detecting the expression of p16 and pKi-67 in CIN and SCC samples. We also investigated whether the expression of p16 has an effect on cell proliferation, which is indicated by the expression of pKi-67.

MATERIALS AND METHODS

A total of 131 cases of various cervical lesions were obtained from the Pathology Department, Universiti Kebangsaan Malaysia Medical Centre (UKMMC). The study was approved by the Ethics Committee of UKMMC. Samples included 43 normal cervices, 40 cases of cervical intraepithelial neoplasia (CIN), and 48 cases of squamous cell carcinoma (SCC) of the cervix. The normal cervical tissues were obtained from women who underwent hysterectomy for benign diseases. The 40 cases of CIN included 16 of low grade squamous intraepithelial lesion (LSIL: CIN 1) and 24 high grade squamous intraepithelial lesions (HSIL: 9 of CIN 2, and 15 of CIN 3). Tumors were

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staged according to the International Federation of Gynecology and Obstetrics (FIGO) criteria [7]. Sixteen patients had FIGO stage I, 17 patients had FIGO stage II, 7 patients had FIGO stage III and 8 patients had FIGO stage IV. All samples obtained included cervical biopsies and surgically resected specimens that were fixed in 10% neutral buffered formalin and embedded in paraffin blocks. For each tissue specimen, several 3 μm sections were cut for hematoxylin and eosin (H&E) and immunohistochemical staining.

IMMUNOHISTOCHEMICAL STAINING

Sequential tissue sections (3 μm) were mounted on poly-L-lysine coated slides. Archival samples were dewaxed by gradual washings in xylene, and then dehydrated in various concentrations of alcohol (100%, 80%, 60% and 40%). Slides were then incubated in 3% hydrogen peroxide in distilled water to quench endogenous peroxidase activity and this was followed by washing in running water. Antigen retrieval was performed by incubating slides in a preheated coplin jar containing Target Retrieval Solution (TRS) at pH 6 (Dako, Denmark) for 20 minutes in a water bath with temperature ranging from 95-99°C. After thermal treatment, the jar with TRS and slides were allowed to cool for 20 minutes at room temperature. Slides were then washed in running water for 3 minutes and were placed in Tris buffer saline (TBS) pH 7.6. Sections were then incubated for 35 minutes with respective primary monoclonal antibodies: anti-p16 (clone E6H4, Dako, Denmark) at a 1:50 dilution, anti-pKi-67 (clone Mib-1, Dako, Denmark) at 1:50 dilution. Reaction products were visualized with biotin-labeled secondary antibody and streptavidin-peroxidase complexes (LSAB® + HRP kit, Dako, Denmark). Diaminobenzidine (DAB) was used as a chromogenic substrate to visualize the antibody-antigen reaction. All sections were then counterstained with hematoxylin and mounted with permanent mountant DPX. Sections were visualized under light microscopy for assessment of immunoreactivity. Cervical carcinoma tissue known to be positive for p16 expression was included as positive control, while tonsil tissue was used as positive control for pKi- 67 immunostaining. Each positive control was consistently positive. Negative controls for p16 and Ki-67 were obtained by substituting the primary antibody with TBS. Negative control sections were unstained.

IMMUNOSTAINING ASSESSMENT

A researcher without knowledge of clinicopathologic data of the samples evaluated all slides for immunostaining in a blind fashion. Confirmation of the diagnosis was done by a pathologist who evaluated the same slides independently. Most of the slides (95%) were classified similarly by both investigators. Discrepancies were resolved by reevaluation and discussion. Cases were included only when there was agreement by both investigators. Staining of p16 was observed in both nuclei and cytoplasm, while immunoreactivity of pKi-67 was observed in the cells with a distinct brown stain confined to the nuclei. In the p16 immunoreactivity assessment, the percentage of positive dysplastic or tumor cells was scored according to the method by de Putte et al. [8] and Ishikawa et al. [9]. When none or less than 5% of dysplastic or tumor cells were stained, it is regarded as negative; when 5-50% cells were stained, it is regarded as 1+; and when more than 50 % cells were stained, it is regarded as 2+. The number of pKi-67 stained nuclei was obtained by counting 200 cells in X400 magnification field for each sample, including all of the epithelial layers. After counting a total of 200 cells, the immunoreactive score was expressed as a percentage of the total cell count or Ki-67 index. The cutoff level to define high and low proliferating tumors was 30% [10, 11].

STATISTICS

Chi-square test was used to evaluate differences in p16 staining, which were analyzed as qualitative variables. However, the mean and standard error of mean (SEM) of the Ki- 67 index were compared using one-way analysis of variance (ANOVA). A p value less or equal to 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 11.0.

RESULTS

Negative staining of p16 was observed in normal squamous epithelium. Diffuse cytoplasmic and nuclear staining was observed in 16/24 (40%) of CIN cases and 30/48 (62.5%) of SCC cases (Figure 1). Expression of pKi-67 was mainly present in the parabasal rather than in the basal layer of normal epithelium (Figure 2a). In CIN 3, pKi-67 staining was diffusely present in the nuclei of dysplastic epithelium and mitotic cells (M) (Figure 2d, e). Diffuse nuclear staining for pKi-67 in most tumor cells of SCC was also observed (Figure 2f). The results of p16 and pKi-67 immunostaining of cervical lesions are summarized in Figure 3. All normal cases were negative for p16 immunostaining. All p16-positive cases in LSIL (25%; 4/16) were scored at 1+. More cases of p16 immunopositivity were observed in HSIL; 8% (2/24) were scored 1+ and 42 % (10/24) were scored 2+. Overexpression of p16 was also observed in majority of the SCC cases (62.5%; 30/48), where 12.5% (6/48) were scored 1+ and 50% (24/48) were scored 2+ (Figure 3a). Majority of pKi-67 immunopositivity in normal cases (96%; 27/28) exhibited low proliferation index with mean index of $10.32 \pm 1.00\%$. High proliferation capacity was observed in LSIL (6%; 1/16), HSIL (17%; 4/24) and SCC (10%; 5/48) cases (Figure 3b). Mean pKi-67 proliferation index in LSIL, HSIL and SCC were $24.75 \pm 4.96\%$, $27.50 \pm 8.50\%$ and 26.00

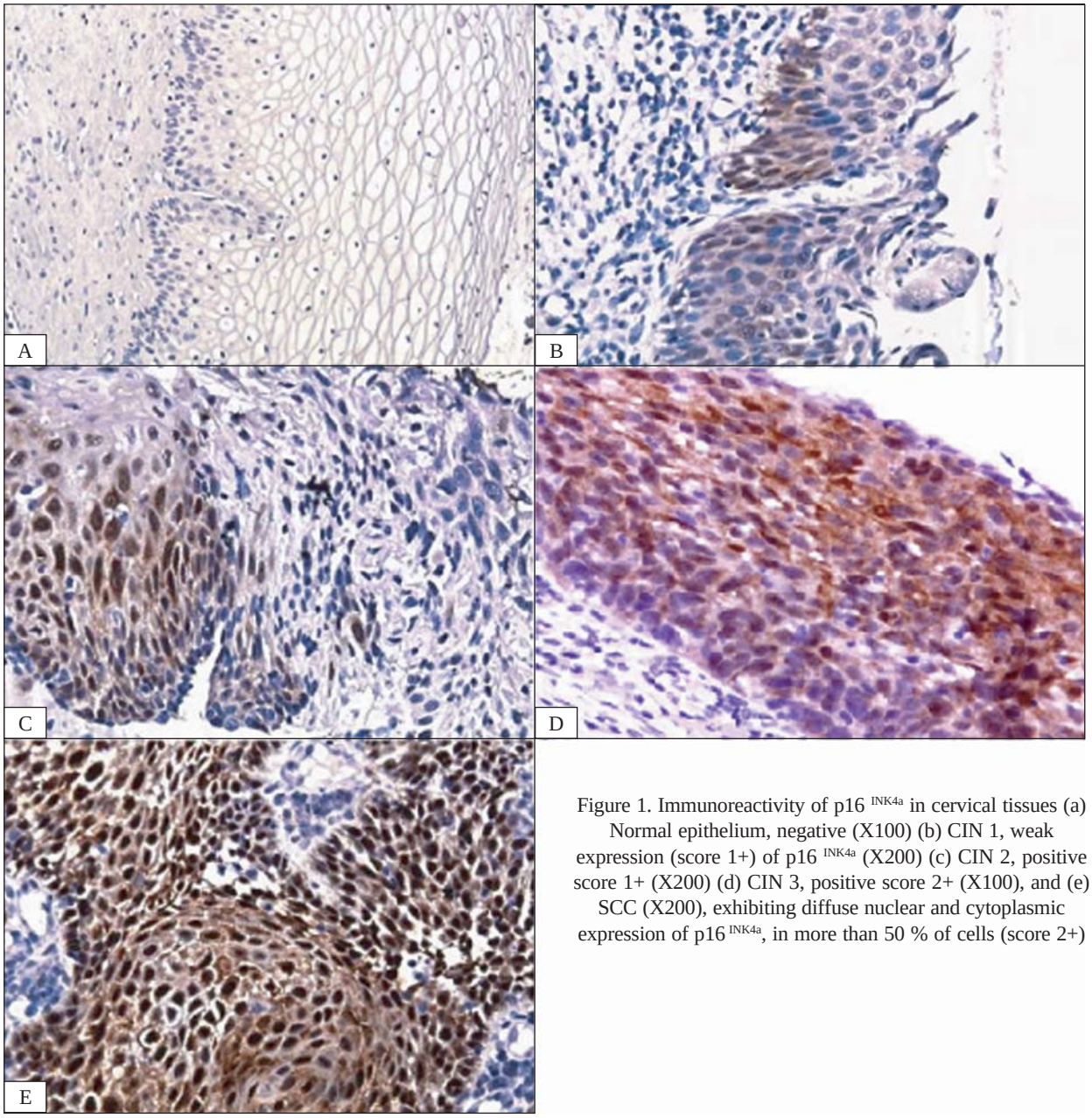

Figure 1. Immunoreactivity of p16 ^{INK4a} in cervical tissues (a) Normal epithelium, negative (X100) (b) CIN 1, weak expression (score 1+) of p16 ^{INK4a} (X200) (c) CIN 2, positive score 1+ (X200) (d) CIN 3, positive score 2+ (X100), and (e) SCC (X200), exhibiting diffuse nuclear and cytoplasmic expression of p16 ^{INK4a}, in more than 50 % of cells (score 2+)

± 6.92%, respectively. HSIL and SCC showed significantly higher Ki-67 index when compared to normal (ANOVA: F = 3.940; p = 0.013). The association between the expression of immunohistochemical markers and grade of the lesions is shown in Table 1. The expression of p16 and pKi-67 proliferation index were significantly associated with the grade of the cervical lesions (p = 0.033 and p = 0.012 respectively). We did not find any significant relationship between overexpression of p16 and pKi-67 proliferation profile ($\chi 2$ = 0.292; p = 0.589) (Table 2).

DISCUSSION

Several previous studies demonstrated strong immunohistochemical expression of p16 in both high risk squamous intraepithelial lesions and SCC of the cervix [1, 5, 12]. One possible explanation of p16 overexpression may be inactivation of pRb by HPV E7 [13]. Although p16 is present in high concentration in these cases, it has no inhibitory effect on the cell cycle because pRb has already been blocked by the E7 oncoprotein. The majority of HPV infections is transient and regresses spontaneously. However, overexpression of p16 has been shown to indicate

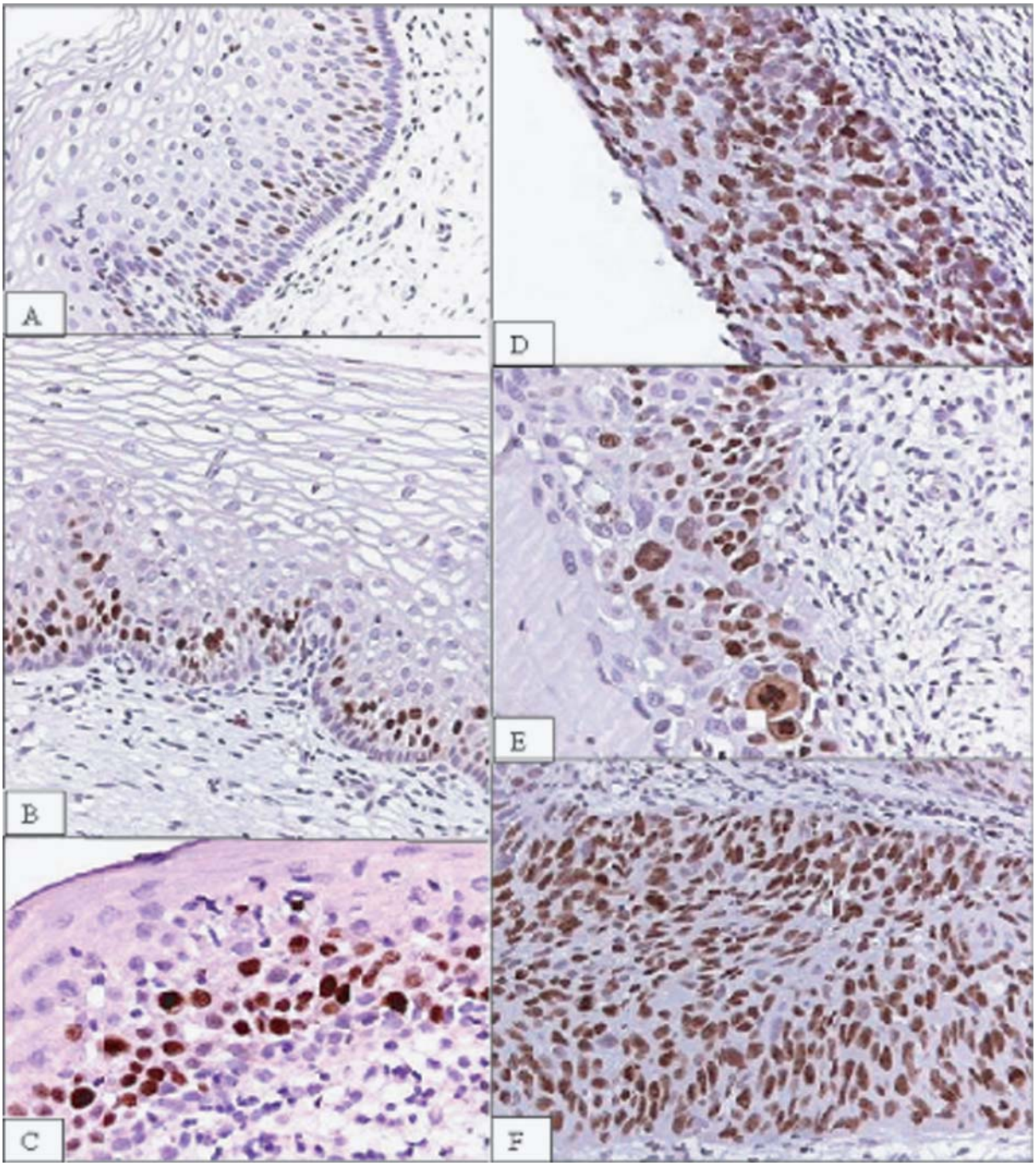

Figure 2. Immunohistochemical expression of pKi-67 in cervical tissues (a) Normal cervical epithelial cells: pKi-67 positive cells were mainly present in the parabasal layer (b) CIN 1 (c) CIN 2 (d) CIN 3: Nuclear staining appears at all levels of the dysplastic epithelium (e) Mitotic cell (*M*) showed immunopositivity of Ki-67, (f) SCC: Intense nuclear staining in tumor cells (X 400 magnification)

already advanced interference of the viral oncoproteins with cellular proteins involved in cell-cycle regulation [14].

A study by Negri et al. [4] found an association between overexpression of p16 in LSIL and high-risk HPV infection. They reported that LSIL cases with diffuse p16 staining had a significantly higher tendency to progress to high-grade lesions compared to p16-negative cases, and hence its expression may serve as a marker of progression in early lesions of the cervix uteri. It has also been suggested that p16 may be a useful adjunct in the diagnosis of CIN in sections of cervical biopsy samples or cervical smears [4, 5, 12]. In our study, p16 expression was not seen in normal cervix or in non-dysplastic tissue. p16 expression was observed in LSIL and the intensity of expression was higher in HSIL and highest in SCC. p16 immunopositivity was seen in both nuclear and cytoplasm of the dysplastic or tumor cells. Our results are in concordance with other studies [4, 12, 13, 15]. According to the report by Murphy

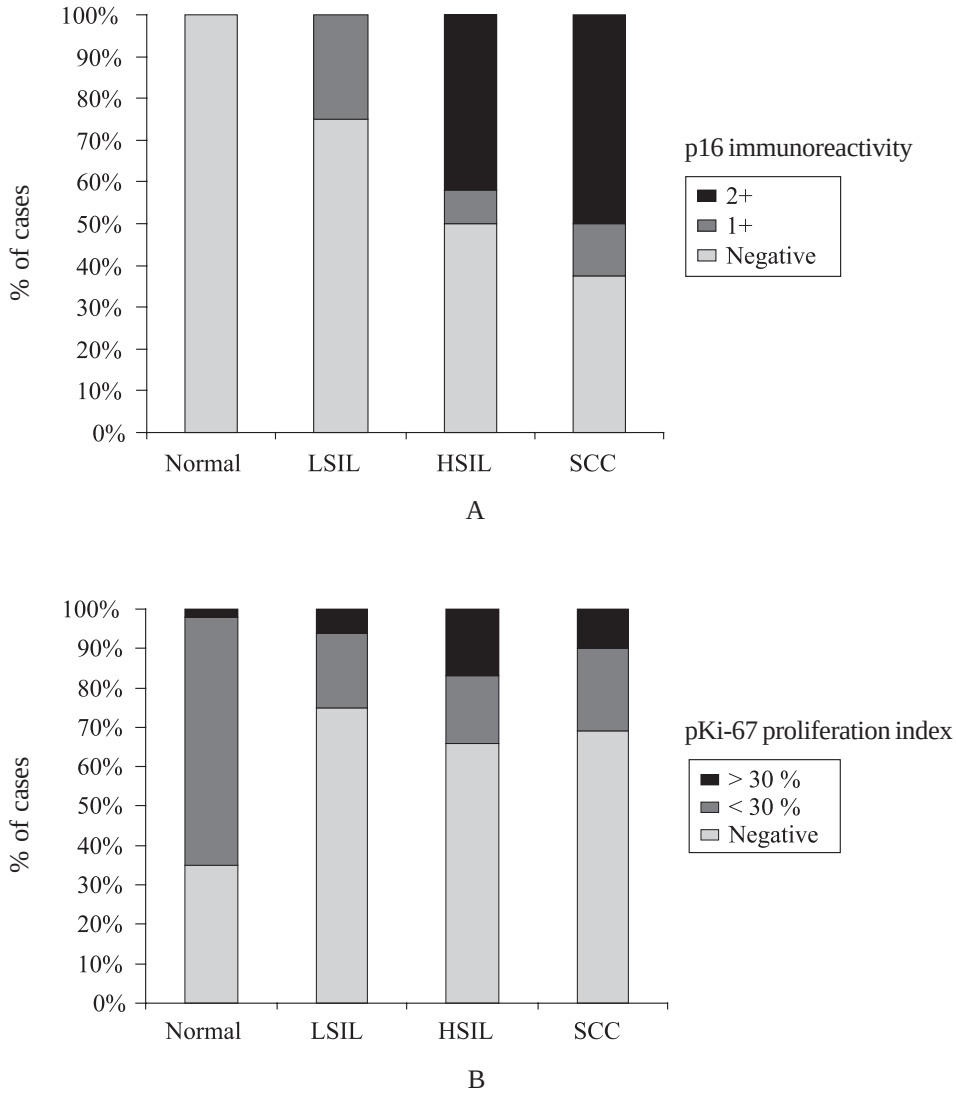

Figure 3. Distribution of p16 expression level (A) and pKi-67 proliferation index (B) according to cervical lesions grades

Table 1. The association between the expression of immunochemicals markers and grade of lesions

	Number of cases (%)			
	Normal	LSIL	HSIL	SCC
p16 immunoreactivity[a]				
Negative	43 (100)	12 (75)	12 (50)	18 (38)
Positive	0 (0)	4 (25)	12 (50)	30 (62)
Total 43 (100)	**16 (100)**	**24 (100)**	**48 (100)**	
pKi-67 proliferation index[b]				
Negative	15 (34)	12 (75)	16 (66)	33 (69)
Low (< 30 %)	27 (63)	3 (19)	4 (17)	10 (21)
High (> 30 %)	1 (3)	1 (6)	4 (17)	5 (10)
Total 43 (100)	**16 (100)**	**24 (100)**	**48 (100)**	

[a] $\chi^2 = 6.832$; $p = 0.033$; [b] $\chi^2 = 10.952$; $p = 0.012$

Table 2. Relationship between p16 expression and Ki-67 proliferation index

	Number of cases		
	Ki-67 proliferation index		Total
	Low (< 30 %)	High (> 30 %)	
p16 Immunoreactivity			
Negative	3	1	4
Positive	14	9	**23**
Total	**17**	**10**	**27**

Note: $\chi^2 = 0.292$; $p = 0.589$

et al. [12], p16 expression was observed to be low at both mRNA and protein level. Hence, p16 may only be detectable when it is upregulated, especially in cervical neoplastic lesions. Overexpression of p16 is usually observed in the nucleus; however its presentation in the cytoplasm may be due to of post-transcriptional modification [12]. Mutation of p16 is a rare event involved in cervical cancer development. Munirajan et al. [16] did not find any mutation or/and deletion of p16 gene in their cervical cancer patients studied. Therefore, inactivation of pRb by HPV E7 protein has been hypothesized to contribute to the overexpression of p16 in cervical lesions. However, there was a limitation in this study as there was no HPV data available for all cases enrolled in this study. Further verification of HPV involvement in the p16/CDK4/cycD1/pRb cell cycle regulatory pathway cannot be elucidated in our study, but what we have demonstrated is the possible potential of p16 as a marker for CIN lesions and cervical cancer as its expression was significantly associated with the increasing grade of the lesions. In our study, only 25% of LSIL, 50% HSIL and 62.5% of SCC cases showed immunopositivity of p16. Our findings differed from that of Murphy et al. [12], where they found all of the CIN and SCC cases studied showed overexpression of p16, with a sensitivity of 99.9 % and a specificity of 100%. Although the number of p16 positively stained cases was lower in our study, we observed the difference in intensity of p16 expression between LSIL, HSIL and SCC. Most of the p16 positive LSIL cases showed low intensity (less than 50% positive cells) whereas a high intensity (more than 50 % positive cells) immunostaining was observed in HSIL and SCC cases. The high intensity of p16 immunoreactivity was found to be distributed at all layers of the dysplastic epithelium and was unique for HSIL. A similar finding was described by Yildlz et al. [17], suggesting the potential use of p16 to differentiate HSIL from LSIL. However due to lack of overexpression p16 in most cases of dysplasia and neoplasia, we suggest that immunostaining of p16 is not an appropriate marker to identify if an individual is at risk for cervical cancer.

On the other hand, the Ki-67 index has been used in several studies to relate with the proliferative capacity of cancer cells. Ki-67 labelling index of less than 10% reflects a benign process [10] particularly inflammation and reactive repair mechanism [18]. It was also found that Ki-67 labeling index of more than 30% was highly predictive of malignant or premalignant lesions [10]. In our study, the majority of normal squamous epithelium (97%) possesses negative or low proliferation activity suggesting that the squamous epithelium controls its cell number by differentiation [19]. The single sample from a normal cervix was found to have a high proliferation index which may be attributed to the reactive type of benign disease. As reported by Walts et al. [20], benign and reactive tissues may show up to 25% of cells stained with pKi-67. High pKi-67 proliferation capacity (Ki-67 index > 30%) was observed during the development of preneoplastic and neoplastic lesions, suggesting a decrease in differentiation but an increase in proliferation as the lesions progress as suggested by Nam et al. [19]. In addition, overexpression of pKi-67 is also regarded as an indicator of biological aggressiveness [21] . In this study, we found that the cell proliferation profile (low or high) is associated with the grade of the cervical lesions. Our data suggest that the progression in the uterine cervix is accompanied by increased cellular proliferation. A similar finding was demonstrated by Herbsleb et al. [22] and Queiroz et al. [1]. This may suggests the possible role of pKi-67 as a biomarker in the grading of cervical lesions.

In our study, there was no significant correlation between p16 expression and expression of pKi-67, suggesting that p16 expression may not have an effect on cell proliferation. This finding was contradictory to other studies which [22, 1, 17] suggested p16 overexpression might induce a progressive increase in cell proliferation. They also identified the co-localization of p16 and pKi-67 in CIN cases and suggested this finding as an independent sign for dysplasia.

In conclusion, we observed an increase in expression of p16 and pKi-67 during the progression of cervical cancer in our cohort of patients, however there was no correlation between these two markers. Both p16 and pKi-67 are essential markers that are useful in the grading of cervical lesions. However, caution is advised on interpreting their expressions, where the lack of p16 and pKi-67 expression does not always represent low risk for progression to HSIL and SCC.

ACKNOWLEDGEMENT

This project was supported by an IRPA grant 06-02-02-0048 from the Ministry of Science, Technology and Innovation of Malaysia. We gratefully acknowledge the staff of the Department of Obstetrics & Gynecology and the Department of Pathology, Universiti Kebangsaan Malaysia Medical Centre for their co-operation in helping with the collection of samples.

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