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

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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 (χ² = 6.832, p = 0.033 and χ² = 10.952; p = 0.012 respectively). There was no significant relationship demonstrated between p16 positivity and Ki-67 proliferation profile (χ² = 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
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 clinico-pathologic 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 ± 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 ± 4.96%, 27.50 ± 8.50% and 26.00
+ 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
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.

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<th>Number of cases (%)</th>
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<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>p16 immunoreactivitya</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>43 (100)</td>
</tr>
<tr>
<td>Positive</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>43 (100)</strong></td>
</tr>
<tr>
<td>pKi-67 proliferation indexb</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>15 (34)</td>
</tr>
<tr>
<td>Low (&lt; 30 %)</td>
<td>27 (63)</td>
</tr>
<tr>
<td>High (&gt; 30 %)</td>
<td>1 (3)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>43 (100)</strong></td>
</tr>
</tbody>
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a $\chi^2 = 6.832; \ p = 0.033$; b $\chi^2 = 10.952; \ p = 0.012$
Ki-67 labeling index of less than 10% reflects several studies to relate with the proliferative capacity of identify if an individual is at risk for cervical cancer. Immunostaining of p16 is not an appropriate marker to most cases of dysplasia and neoplasia, we suggest that from LSIL. However due to lack of overexpression p16 in 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

<table>
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<th>p16 Immunoreactivity</th>
<th>Ki-67 proliferation index</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>Low (&lt; 30 %)</td>
<td>High (&gt; 30 %)</td>
</tr>
<tr>
<td>Negative</td>
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<td>1</td>
</tr>
<tr>
<td>Positive</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
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Note: $\chi^2 = 0.292; p = 0.589$
ACKNOWLEDGEMENT

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REFERENCES


