Immunohistochemical Localization of Cdc6 in Squamous and Glandular Neoplasia of the Uterine Cervix

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- Context.—Cdc6 has been extensively studied as a marker for cellular proliferation that is expressed during the normal cell cycle. Recent studies indicate that Cdc6 may be a marker for cervical intraepithelial neoplasia (CIN) and carcinoma; however, the histologic distribution of Cdc6 has not been explicitly defined. Expression of Cdc6 in the endocervical mucosa also remains unexplored.

- Objective.—The goal of the current study was to evaluate the distribution of Cdc6 protein, MIB-1 protein, and human papillomavirus (HPV) DNA in a broad range of cervical tissues, including normal, potentially premalignant, and malignant lesions of the ectocervical and endocervical mucosa.

- Methods.—We used an indirect immunoperoxidase method to stain formalin-fixed, paraffin-embedded tissues and frozen tissues, including biopsy and hysterectomy specimens, for Cdc6 and MIB-1 proteins, and we used in situ hybridization to detect HPV DNA in a subset of cases.

- Results.—Cdc6 staining was exclusively nuclear and was present in both squamous and glandular epithelial cells of histologic sections. Cdc6 staining was rarely present in specimens of normal cervical squamous mucosa (2/84, 2.4%) or in specimens with squamous metaplasia (3/59, 5.1%) and was not detected in normal endocervical glands (0/84). Staining was present in most cases of CIN I (31/48, 65%). Staining was present in the majority of cases of CIN II (25/28, 89%) and in all cases of CIN III (36/36) and squamous cell carcinomas (34/34). The proportion of cells staining for Cdc6 increased with the grade of dysplasia, and the proportion of stained cells in squamous cell carcinomas was similar to that in lesions of high-grade dysplasia. Cdc6 staining was present in the majority of cases in glandular lesions including adenocarcinoma in situ (11/14, 79%) and adenocarcinoma (8/10, 80%). The histologic distribution of Cdc6-immunoreactive cells was similar to that of cells with a strong signal for HPV DNA, but Cdc6 protein and HPV DNA did not colocalize at the level of individual cells.

- Conclusion.—Cdc6 expression is a marker for high-grade cervical squamous and glandular dysplasia and carcinoma and is associated with HPV infection. The mechanistic basis of the association between HPV infection and Cdc6 immunopositivity remains to be determined but may represent either up-regulation of Cdc6 expression or stabilization of the Cdc6 protein.

(Arch Pathol Lab Med. 2002;126:1163–1168)
Cdc6 is essential for the initiation of DNA replication and, in combination with other regulatory proteins, also limits cells to a single round of synthesis per cell cycle. Cdc6 seems to be part of the prereplication complex and is required for the initiation of DNA synthesis but is released from the DNA-protein replicative complex during quiescence and differentiation. The influence of HPV-associated oncoproteins on the regulation of Cdc6 expression has not yet been well characterized.

Previous studies have reported that Cdc6 is expressed in both benign cervical mucosa and in potentially pre-neoplastic and malignant lesions of the cervix. Specific details regarding the histologic distribution of Cdc6 in benign and malignant cervical tissues, however, have not been explicitly described. Thus, the purpose of the current study was to confirm the hypothesis that Cdc6 expression is a marker for cervical squamous and glandular dysplasia and carcinoma and to correlate the distribution of Cdc6-positive cells with the distribution of cells containing HPV DNA. In addition, we assessed the correlation between the distribution of Cdc6 and the distribution of a marker of cell proliferation, MIB-1.

MATERIALS AND METHODS

Tissue Samples

We obtained surgical cervical specimens from the archival collections of the Department of Pathology at the University of Colorado Health Sciences Center. A total of 397 separate diagnoses were made on 191 samples obtained from 187 different patients. We selected the cases to include examples of unequivocal histologic diagnoses in each category, with sufficient residual tissue in the archival tissue blocks to ensure an adequate sample for immunohistochemical analysis. We excluded from analysis cases in which the original lesions identified by hematoxylin-cosin staining were scant or no longer present on subsequent study tissue sections. The final set of study cases included samples of normal cervical squamous mucosa (84), squamous metaplasia (59), CIN I (48), CIN II (28), CIN III (36), and invasive squamous cell carcinoma (34). Glandular tissues included normal endocervical mucosa (84), adenocarcinoma in situ (10), and invasive adenocarcinoma (10). We selected routinely processed formalin-fixed, paraffin-embedded tissue blocks and prepared 5-μm serial sections from the cut surface of the blocks. Before deparaffinizing the sections, we baked them overnight at 60°C.

Immunohistochemical Analysis

Tissue sections were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide for 10 minutes. Antigen retrieval was performed by immersing tissue sections in 0.1 M citrate buffer (pH 6.0) and heating them for 10 minutes in a Decloaking Chamber (Biocare Medical, Walnut Creek, Calif). After antigen retrieval, endogenous biotin in the tissue block was blocked using the Biotin Blocking System (Dako Corporation, Carpinteria, Calif). Sections were subsequently incubated with a mouse monoclonal antibody that is specific for Cdc6 (NeoMarkers, Fremont, Calif). Sections were incubated with the Cdc6 primary antibody overnight in a humidified chamber at 4°C. The MIB-1 antigen was localized by incubating sections with a commercially available kit and protocol (Kreatech Diagnostics, distributed by Zymed Laboratories, Inc, San Francisco, Calif) including proteolytic treatment; hybridization with HPV DNA probes for types 16/18, 6/11, and 31/33; detection; and staining. Positive and negative control sections were processed for each case as indicated in the test kit protocol. Matched serial sections from HPV DNA–positive cases were processed for Cdc6 and MIB-1 immunohistochemistry, as described previously.

RESULTS

Staining for Cdc6 was present in 31 of 48 cases of CIN I, 25 of 28 cases of CIN II, 36 of 36 cases of CIN III, and 34 of 34 cases of squamous cell carcinoma. Cdc6 staining was rarely present in cases of normal cervical squamous mucosa (2/84) or in cases of squamous metaplasia with or without reactive changes (3/59). In positive cases, staining was exclusively nuclear and heterogeneously distributed (Figure 1, A, C, E, and G). Within areas of dysplasia that showed Cdc6 expression, nuclear staining was preferentially present in the intermediate and upper layers of the lesional mucosa. The lower portions of the intermediate cell layers contained few positive cells, and staining was rarely present in basal epithelial cells.

The proportion of cells that stained for Cdc6 increased progressively with increasing grade of dysplasia (Table). The mean Cdc6 staining score was 1.08 in cases of CIN I (range, 0–4; median, 1.5), 1.89 in cases of CIN II (range, 0–4; median, 2), 3.33 in cases of CIN III (range, 1–4; median, 4), and 3.2 in cases of squamous cell carcinoma (range, 1–4; median, 4). Nuclear staining also correlated with histologic evidence of HPV infection. Two of the 3 cases of CIN I with the greatest proportion of Cdc6-stained nuclei showed unequivocal features of HPV infection, including acanthosis, papillomatosis, and marked koilocytic atypia. In contrast, cases that were classified as CIN I based only on disordered maturation of the lower third of the squamous mucosa but that lacked convincing HPV-associated histologic changes were usually negative for Cdc6. In the few cases in this diagnostic category that were Cdc6 positive but lacked cytologic evidence of HPV infection, Cdc6 staining was restricted to the lower third of the squamous epithelium.

The preferential expression of Cdc6 in the upper layers of the dysplastic lesional epithelium raised the possibility that Cdc6 expression could be related to HPV viral replication. To investigate this possibility, we correlated the distribution of Cdc6 expression with the distribution of HPV DNA staining in matched serial sections of 7 HPV-

<table>
<thead>
<tr>
<th>Histologic Diagnosis</th>
<th>Cdc6 Score, Mean (Range)</th>
<th>Median</th>
<th>No. of Cdc6-Positive Cases/Total No. of Cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal squamous mucosa</td>
<td>0 (0-1) [0]</td>
<td>[0]</td>
<td>2/84 (2.4)</td>
</tr>
<tr>
<td>Squamous metaplasia</td>
<td>0.01 (0-1) [0]</td>
<td>[0]</td>
<td>3/59 (5.1)</td>
</tr>
<tr>
<td>CIN I</td>
<td>1.08 (0-4) [1.5]</td>
<td>[1.5]</td>
<td>31/48 (65)</td>
</tr>
<tr>
<td>CIN II</td>
<td>1.89 (0-4) [2]</td>
<td>[2]</td>
<td>25/28 (89)</td>
</tr>
<tr>
<td>CIN III</td>
<td>3.33 (1-4) [4]</td>
<td>[4]</td>
<td>36/36 (100)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>3.2 (1-4) [4]</td>
<td>[4]</td>
<td>34/34 (100)</td>
</tr>
<tr>
<td>Normal endocervical glands</td>
<td>0 (0) [0]</td>
<td>[0]</td>
<td>0/84 (0)</td>
</tr>
<tr>
<td>Adenocarcinoma in situ</td>
<td>2.3 (0-4) [1.5]</td>
<td>[1.5]</td>
<td>11/14 (79)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>2.14 (0-4) [2.5]</td>
<td>[2.5]</td>
<td>8/10 (80)</td>
</tr>
</tbody>
</table>

*CIN indicates cervical intraepithelial neoplasia.
† Staining for Cdc6 was scored as 0 (negative), 1 (<1% of nuclei), 2 (1% to 5% of nuclei), 3 (>5% to 10% of nuclei), or 4 (>10% of nuclei).
‡ Positive cases are those with a Cdc6 score of ≥1.

positive cases of CIN. Cdc6-positive nuclei tended to be located lateral to or immediately beneath cells that were strongly positive for HPV DNA (Figure 2). Although nuclear staining for Cdc6 protein and HPV DNA did not colocalize at the level of individual cells, there was a general correlation between the histologic distributions of these markers.

We performed immunohistochemical staining for MIB-1 on a subset of cases of normal cervical mucosa, CIN, and squamous cell carcinoma to compare the distribution of MIB-1 with the distribution of Cdc6. Intense MIB-1 nuclear staining was present in all cases, and its distribution differed from that of Cdc6 (Figure 1, B, D, F, and H). In contrast to Cdc6 staining, MIB-1 staining was present in the parabasal cells of normal squamous mucosa. In cases of CIN I, MIB-1–positive nuclei were present in the lower third of the epithelium, although in cases with marked HPV-associated nuclear atypia, MIB-1 staining extended...
into the upper third of the epithelium. MIB-1 staining in cases of CIN III was distributed throughout the full thickness of the lesional mucosa, in contrast to the preferential distribution of Cdc6 in the upper layers. In squamous cell carcinomas, MIB-1 staining tended to be most prominent at the periphery of tumor cell nests, but Cdc6 staining was more concentrated in the central regions of infiltrative clusters of tumor cells (Figure 1, G and H).

Cdc6 staining was also present in 11 of 14 cases of adenocarcinoma in situ and in 8 of 10 cases of adenocarcinoma. In addition, Cdc6-positive nuclei tended to show basal polarity in cases of adenocarcinoma in situ, whereas Cdc6-positive nuclei were more randomly distributed throughout the lesional epithelium in invasive adenocarcinomas (Figure 3, A and C, respectively). The proportion of stained nuclei in cases of adenocarcinoma in situ was similar to that in cases of adenocarcinoma in situ (Table). The mean Cdc6 staining scores were 2.3 in cases of adenocarcinoma in situ (range, 0–4; median, 1.5) and 2.14 in cases of adenocarcinoma (range, 0–4; median, 2.5). MIB-1 nuclear staining was present extensively throughout the lesions of both adenocarcinoma in situ and adenocarcinoma, with all areas of the lesions equally involved, but did not correlate with Cdc6 expression at the level of individual cells (Figure 3, B and D). Normal endocervical cells and stromal cells were uniformly negative for Cdc6 (0/84 cases).

**COMMENT**

We detected immunohistochemical staining for Cdc6 in a wide range of lesions of the cervical mucosa. Although rare Cdc6-positive cells were present in normal mucosal tissues or in squamous metaplasia, positive cells were present at least in scattered lesional cells in a high proportion of CIN I lesions, in at least some dysplastic cells in almost all CIN II/III lesions, and in all squamous cell carcinomas. In addition, Cdc6 expression was seen in the great majority of cases of endocervical adenocarcinoma in situ and adenocarcinoma. Thus, Cdc6 expression seems to be a marker of both squamous and glandular neoplasia of the cervical mucosa.

Although previous studies have reported that a high proportion of cervical dysplastic squamous cells stain for Cdc6, in the current study, Cdc6 staining was present in only a relatively small proportion of lesional cells. Thus, despite the fact that Cdc6 staining correlates with the histologic diagnosis and is a marker for cervical dysplasia and squamous cell carcinoma, it is not detectable in a high proportion of lesional cells. However, similar to the pattern noted in previous reports, the proportion of stained cells was low in benign lesions and increased with increasing grade of squamous dysplasia. The proportion of Cdc6-positive cells in squamous cell carcinomas, however, was similar to that in lesions of high-grade dysplasia.
The possible causes for the discrepancy in the proportion of stained cells observed between the current study and previous studies\textsuperscript{17,18} include differences in the Cdc6 antibodies used; differences in the staining characteristics of frozen sections versus sections of fixed, paraffin-embedded tissues; and differences in the staining protocols. Although previous studies used polyclonal rabbit antibodies to Cdc6 that were generated by immunization with His-tag fusion proteins,\textsuperscript{17,18} we used commercially available mouse monoclonal antibodies. It is possible that the Cdc6 antigenic site in the DNA-protein replicative complex is not recognized by the monoclonal antibody that we used in the current study, but that other antigenic sites in the bound form of Cdc6 are detectable with the polyclonal antibodies that were previously described.\textsuperscript{17–19} The distribution of Cdc6 staining has been reported to be similar in sections of formalin-fixed, paraffin-embedded tissue blocks and frozen sections.\textsuperscript{17,18} Similarly, we have confirmed an identical pattern of Cdc6 staining in sections of formalin-fixed tissues and in corresponding frozen sections (L.B., C.G., and K.R.S., oral communication, November 2000). Thus, differences in tissue fixation are an unlikely explanation for the differences between the current observations and those of previously reported studies.\textsuperscript{17,18} The staining protocol that we used in the current study included antigen retrieval by heating denaturation in citrate buffer, followed by an indirect avidin-biotin-based immunoperoxidase method. This protocol is similar to that reported in previous studies,\textsuperscript{17,18} and therefore methodologic differences are an unlikely explanation for the differences between the current results and those previously reported by other groups. Thus, differences in tissue fixation or immunohistochemical staining protocols are unlikely to explain the lower proportion of stained cells observed in the current study compared with that observed by Freeman et al\textsuperscript{17} and Williams et al.\textsuperscript{18} Alternatively, the use of monoclonal versus polyclonal antibodies is a likely explanation for this discrepancy. Although Cdc6 expression has been studied in cervical squamous epithelial lesions, its expression in premalignant and malignant glandular epithelial tissues has not been previously characterized. The current study shows that Cdc6 is expressed in a high proportion of cases of both adenocarcinoma in situ and in invasive endocervical adenocarcinoma. Thus, Cdc6 expression may be a general marker of cervical neoplasia, and its expression is not restricted to cells of either squamous or glandular differentiation. This observation suggests that Cdc6 staining could have a broader role as a diagnostic adjunct in the histologic evaluation of cervical neoplasia.

Although Cdc6 expression is a specific marker for cell division, the proportion of cells staining for Cdc6 was much lower than the proportion of cells staining for MIB-1 in both squamous and glandular lesions. Differences in the proportion of cells that stain for Cdc6 and MIB-1 may reflect temporal and mechanistic differences in the expression of these proteins throughout the cell cycle. Although Ki-67 is present throughout the cell cycle, including the S, G2, and M phases, and in some instances late G1 phases,\textsuperscript{20–22} the expression of Cdc6 is restricted to the G1 phase.\textsuperscript{23} Differences in the proportion of cells that stain for Cdc6 protein and the MIB-1 antigen could also reflect differences in the rate of intracellular turnover of these proteins. Although the MIB-1 antigen has a reported half-life of 60 to 90 minutes,\textsuperscript{24,25} the half-life of unbound nuclear Cdc6 is only about 15 to 30 minutes in the early G1 phase.\textsuperscript{23} Thus, the more restricted expression of Cdc6 and the higher rate of intracellular turnover would predict that the proportion of cycling cells containing Cdc6 would be lower than that containing MIB-1 at any point in time.

To the best of our knowledge, this study represents the first reported observation of the association between HPV DNA distribution and Cdc6 protein distribution. Staining of paired serial sections showed a general correlation between HPV DNA and Cdc6 distributions at the histologic level. However, at the level of individual cells, we did not observe a direct correlation. The increased expression of Cdc6 in areas with high levels of HPV DNA suggests that Cdc6 might either contribute to viral DNA replication or reflect activation of genomic DNA replicative processes by HPV-associated oncoproteins. Further studies may help to define the mechanisms by which HPV-associated oncoproteins may affect Cdc6 expression in cervical neoplasia.

In summary, Cdc6 is a specific marker for neoplasia in both squamous and glandular lesions of the cervical mucosa. The proportion of cells that stain for Cdc6 could relate to both antigenic variability and antibody specificities. Further studies are indicated to elucidate the mechanisms underlying the observed association between Cdc6 expression and HPV infection. The possible role of Cdc6 expression as a diagnostic or prognostic marker for cervical neoplasia remains to be determined. Expression of Cdc6 could be used as a molecular marker for high-grade dysplasia in diagnostic cervical cytopathology. In this context, Cdc6 expression might be used to triage cases with low-grade or equivocal cytologic diagnoses to identify underlying high-grade lesions. Further studies are required to evaluate the correlation of Cdc6 immunopositivity with cervical cytologic diagnoses both for patients at high risk and for patients in general screening populations.

References


