Study of Proliferation Markers in Normal and Diseased Uterine Cervix

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Abstract
Carcinoma of the cervix is one of the most common malignancies. Papanicolaou (Pap) smear tests have reduced mortality by up to 70%. Nevertheless their interpretation is notoriously difficult with high false-negative rates and frequently fatal consequences. We have addressed this problem by using affinity-purified antibodies against human proteins that regulate DNA replication, namely Cdc6 and Mcm5. Our materials were obtained from 100 smears and 60 cervical biopsies specimens taken at colposcopy clinics. Twenty-seven biopsies showed high-grade squamous intraepithelial lesions (HSILs), and 14 showed low-grade squamous intraepithelial lesions (LSILs). Normal cervical tissue was obtained from age-matched patients undergoing hysterectomy for diseases unrelated to the cervix (n = 19). These antibodies were applied to sections and smears of normal and diseased uterine cervix by using immunohistochemical technique to detect abnormal precursor malignant cells. Antibodies against Cdc6 and Mcm5 stain abnormal cells in cervical smears and sections with remarkably high specificity and sensitivity. Proliferation markers Ki-67 and proliferating cell nuclear antigen are much less effective. The majority of abnormal precursor malignant cells are stained in both low-grade and high-grade squamous intraepithelial lesions. Immunoestaining of cervical smears can be combined with the conventional Pap stain so that all the morphological information from the conventional method is conserved. Thus antibodies against proteins that regulate DNA replication can reduce the high false-negative rate of the Pap smear test.
Introduction
Carcinoma of the cervix is the eighth most common malignancy of women in the United Kingdom and the most common malignancy in women under 35 years of age [1]. In the developing world it is the most common malignancy in women between the ages of 35–45 years with an estimated 437,000 new cases each year [2]. The majority of cases represent squamous cell carcinoma and are strongly associated with infection by high-risk types of human papilloma virus (HPV), such as 16, 18, and 31 [3]. Cervical carcinoma is amenable to prevention by population screening, as it evolves through well-defined noninvasive intraepithelial stages, which can be distinguished morphologically [4]. Squamous intraepithelial abnormalities may be classified by using three-tier (CIN) or two-tier (Bethesda) systems. As classified by the Bethesda system, low-grade squamous intraepithelial lesions (LSIL), corresponding to CIN1 and cervical HPV infection, generally represent productive HPV infections with a relatively low risk of progression to invasive disease [5]. High-grade squamous intraepithelial lesions (HSIL), corresponding to CIN2 and CIN3, show a higher risk of progression than LSIL though both LSIL and HSIL are viewed as representing a potential precursor of malignancy. The introduction of the Papanicolaou (Pap) smear test [6] to identify these precursor lesions has proved to be the most successful public health measure introduced for the prevention of cancer and has proven highly effective in reducing cervical cancer mortality and morbidity rates. The Pap test samples approximately 500,000–600,000 superficial surface cells from the epithelium of the cervix (exfoliative cytology). Smear preparations are made from these samples and screened for the presence of precursor malignant (dysplastic) cells by using morphological criteria. If detected early, cervical cancer is easily treated.

However, despite the introduction of mass screening programs [7]. The failure of the Pap test to eradicate cervical cancer emphasizes the limitations of this screening method. It is prone to errors at all levels, including taking the smear, identifying and interpreting abnormalities in the cytological specimen, and doing inadequate follow-up procedures [8]. Consequently, high numbers of false-negative results (20–40%) are associated with this test [7]. This failure partly reflects the subjectivity of cytological diagnosis and the limited time available for screening each slide because of excessive workloads. Hence abnormal cells are missed, especially if the proportion of abnormal cells in the smear is low because of inadequate sampling. In this study we have identified proliferation markers proteins as markers for cytological assessment that can improve the detection efficiency for precursor malignant cells in the Pap smear test. This detection method can be combined with Pap stain to give an immunoenhanced Pap smear test.

Established cell proliferation markers such as Ki-67 and proliferating cell nuclear antigen (PCNA) have not been useful for cervical smear analysis. We have examined two proteins involved in the regulation of DNA replication, namely Cdc6 and Mcm5. These proteins are sequentially assembled into a prereplicative complex or “replication license” that is essential for the initiation of DNA replication. Disassembly of this complex during replication serves as a ratchet to prevent reinitiation of replication within a single cell cycle [9–11]. These highly conserved proteins appear to be essential for initiation of DNA replication in all types of eukaryotic cells tested so far [11].

We report here that antibodies against PCNA (proliferating cell nuclear antigen) are powerful markers of cell proliferation and that they have major diagnostic potential for detecting abnormal precursor malignant cells in cervical smears and biopsies. Immunostaining with these antibodies can be superimposed over the conventional Pap stain so that the
advantages of both methods can be exploited.

**Materials and Methods**
The materials were obtained from 100 smears and 60 cervical biopsies specimens taken at colposcopy clinics in Department of Gynecological & Obstetric and Hilla Gynecological & Obstetric Teaching Hospital, Medical College, Babylon University, Iraq. Twenty-seven biopsies showed HSILs, and 14 showed LSILs. Normal cervical tissue was obtained from age-matched patients undergoing hysterectomy for diseases unrelated to the cervix (n = 19). Tissue from each specimen was fixed in formalin and processed to paraffin. In each case smears and/or cervical biopsies also were obtained for routine processing.

**Immunohistochemical Staining.** Serial sections (5 μm) were fixed for 10 min. Endogenous peroxidase activity was quenched by incubation in 0.6% hydrogen peroxide/100% methanol for 30 min. Sections were washed in tris-buffered saline (TBS) and blocked overnight with 10% goat serum in TBS. Primary antibodies were diluted [PCNA 1:100, Ki-67 (Dako) 1:20, Mcm5 1:200, and Cdc6 1:400] in TBS containing 1% BSA, and 100 μl was added to each section [12,13]. Incubation was at 4°C in a humidified chamber. The slides then were washed in TBS, followed by biotinylated goat anti-rabbit secondary antibody (Dako) at 1:500 in TBS containing 1% BSA for 30 min at room temperature. After washes in TBS, streptavidin horseradish peroxidase (HRP, Dako) was added. After additional washes, the presence of HRP was detected with the substrate diaminobenzidine (Sigma). The reaction was stopped by rinsing in water, and slides were lightly counterstained with hematoxylin, dehydrated through graded ethanols, and cleared in xylene. Coverslips were applied with DPX mounting medium.

**Cervical Smear Preparation.** Fresh smears were fixed for 5 min in acetone/methanol (50:50). For immunostaining, endogenous peroxidase activity was quenched , cells were permeabilized with 4 mM sodium deoxycholate for 10 min, washed with TBS and blocked with 10% goat serum in TBS. Primary antibodies were diluted as above in TBS containing 1% BSA and incubated at 4°C. Washing steps and the secondary antibody step were performed essentially as described above for sections. Smears were either lightly counterstained with hematoxylin or standard Pap stain [6] before mounting. Appropriate controls were performed in all systems, they gave negative immunostaining.

**Statistic analysis**
The association between various proliferation markers in both low-grade and high-grade squamous intraepithelial lesions was assessed using the Wilcoxon rank sum test and the paired \( t \)-test. Two sided \( P \) values were calculated and values <0.05 were considered significant.

**Results**
Antibodies against proliferation markers specifically detect abnormal precursor malignant cells in cervical tissue sections and cervical smears (Fig1 - 3). These results suggested that anti-Cdc6 and anti-Mcm5 antibodies, might provide specific markers for cells in the proliferative cycle. To determine whether these proliferation markers can be used to detect abnormal precursor malignant cells in cervical smears we tested affinity-purified polyclonal antibodies raised against human Cdc6 and Mcm5 proteins on serial sections of normal and abnormal human uterine cervix and on cervical smears. Immunostaining with these antibodies was compared with the staining pattern obtained with conventional proliferation markers PCNA and Ki-67. Although all were expected to perform as proliferation markers, striking differences were observed in their potential diagnostic value. The staining pattern of tissue sections of normal cervix was similar with all four antibodies (Fig. 1). All showed staining of the basal proliferating layers of
the cervical squamous epithelium but did not stain the superficial differentiating cells. However, marked differences were observed between antibodies when applied to tissue sections of both HSIL and LSIL. Specifically, antibodies against Cdc6 and Mcm5 were found to stain a much higher proportion of abnormal cells than antibodies against Ki-67 or PCNA in both grades of lesion. Table 1 shows each proliferation markers values as calculated from five different sections and the results of the Wilcoxon rank sum test (P values are two-sided: PCNA vs Mcm5 P < 0.01; PCNA vs Cdc6 P < 0.01; Ki-67 vs Mcm5 P < 0.01; Ki-67 vs Cdc6 P < 0.01; PCNA vs Mcm5 P < 0.05; PCNA vs Cdc6 P < 0.05; Ki-67 vs Mcm5 P < 0.05; Ki-67 vs Cdc6 P < 0.05.).

Although full thickness staining of the epithelium was observed with all antibodies for HSILs, only antibodies against Cdc6 and Mcm5 stained both the basal and superficial layers in LSILs. Interestingly, koilocytes, cells that show HPV cytopathic effect, stained strongly for proliferation markers. This high degree of sensitivity using antibodies against Mcm5 and Cdc6 was accompanied by a high degree of specificity; adjacent normal superficial ectocervical, metaplastic, stromal, and inflammatory cells were not stained. Negative immunostaining with anti-Mcm5 and anti-Cdc6 antibodies also was observed for surface and glandular endocervical epithelium. The specificity of immunostaining with anti-Mcm5 or anti-Cdc6 antibodies was confirmed by blocking with the proteins used to raise the antibodies. Identical patterns of immunostaining were observed within each category during examination of 60 biopsies (27 HSIL, 14 LSIL, and 19 normal), indicating that antibodies against Mcm5 or Cdc6 are of major potential diagnostic value for detecting abnormal precursor malignant cells.

Cervical smear tests sample cells from superficial surface layers of the cervical epithelium normally should contain only nonproliferating differentiated cells. Results with cervical tissue specimens suggest that anti-Mcm5 and anti-Cdc6 antibodies may be important for diagnosis in the cervical screening test. Cells obtained by cervical smear examination from normal, metaplastic, and squamous intraepithelial lesions were examined with all antibodies. Normal ectocervical, endocervical, metaplastic, and inflammatory cells showed no immunostaining with any of the antibodies tested. However, as in the case of the cervical tissue sections, marked differences were seen in the immunostaining of abnormal cells derived from LSILs or HSILs. Although Ki-67 and PCNA showed nuclear staining of only a minority population of the abnormal precursor malignant cells, Mcm5 and Cdc6 showed strong immunostaining of the majority of the abnormal cells in the smear. High specificity and sensitivity were observed during examination of 100 smears. This high degree of sensitivity was observed for all subtypes of SILs, including immature (basaloid type), mature (keratinizing), and metaplastic as well as for abnormal cells in four cases of glandular neoplasia/adenocarcinoma. Thus the sensitivity and specificity of these antibodies for detection of abnormal precursor malignant cells in tissue sections also is observed in cervical smear preparations.

An immunoenhanced Pap test shows increased efficiency of detection of abnormal cells in the cervical smear test. Table 2 shows that of 31 cases assessed as positive for LSIL, HSIL, or endocervical adenocarcinoma, in 25 of which a histological diagnosis was made, all 31 also were classified as positive by immunoenhanced Pap test.

**Discussion**

The Pap smear test [6] has been extremely effective in reducing cervical cancer rates. However, this complex test is prone to errors at multiple levels, resulting in a high false-negative rate of 20–40% [7]. A major
factor contributing to the false-negative rate is the failure of detection of abnormal precursor malignant cells [8]. This failure is most likely to occur in routine screening where there are a low number of abnormal cells caused by sampling error combined with insufficient screening time as a result of excessive workloads. Here we demonstrate that antibodies against DNA replication regulatory proteins [14-16] can facilitate the detection of abnormal cells and help to reduce the high false-negative rate.

Many oncogenes encode growth factors, receptors, or proteins of signal transduction pathways [17]. These pathways are inherently redundant, so that no single oncogene can be a reliable marker for all neoplastic cells. However, signaling pathways converge at the point of initiation of DNA replication. Minichromosome maintenance proteins (Mcm) and Cdc6 are essential for the key regulatory step of initiation of DNA replication in all eukaryotes investigated so far [9–11, 18, 19]. Observations have shown that these proteins are present throughout the cell cycle of proliferating cells, but not in nonproliferating quiescent cells [14]. We have tested antibodies against Mcm5 and Cdc6 to distinguish abnormal proliferating cells from a background of normal, nonproliferating differentiated cells in cervical smears.

Our data obtained from 100 smears and 60 biopsies show that abnormal precursor malignant cells of SILs (dysplastic cells) can be detected with high specificity and sensitivity by immunohistochemical staining (Figs. 2, 3,) using antibodies against Cdc6 or Mcm5. One of the reasons these antibodies appear to be superior for diagnosis compared with antibodies against PCNA and Ki-67 relates to the preservation of these antigens in cytological material. Although similar results have been obtained by other investigators [19,20] and when comparing antibodies against PCNA with either anti-Mcm5 or anti-Mcm7 antibodies on formalin-fixed, paraffin wax-embedded tissue sections, we show here that the sensitivity for Mcm5 and Cdc6 is much higher than for PCNA or Ki-67 when applied to cervical smears.

To date dysplastic cells have been characterized by the classical morphological features of maturation arrest and associated cytological abnormalities. We suggest that dysplastic cells can alternatively be recognized by their persistence in the cell cycle compared with normal epithelial cells that exit the cell cycle during maturation and differentiation. We have exploited this difference by using antibodies directed against proteins of the prereplicative complex, which are characteristic of cycling, but not quiescent cells, to provide a powerful complementary test that can be combined with the standard Pap stain test. Our results show that this immunoenhanced Pap test has the potential to increase the detection efficiency for precursor malignant cells in the cervical smear test for the most clinically significant abnormalities. It is possible that some of the more unstable and immature phases of squamous metaplasia may be detected in the immunoenhanced test because these cells are immature and may still be in cell cycle. However, these cells are identified only in a minority population of cervical smears, and they can be readily identified by morphological criteria using the Pap stain.

**Conclusion**

A modification of the standard Pap cervical smear test for detection of abnormal precursor malignant cells was reported. The immunoenhanced Pap test identifies abnormal cells both in terms of morphological and functional criteria, the former by using antibodies against proteins of the prereplicative complex, characteristic of cycling cells. This complementary approach should markedly improve the accuracy, precision, and sensitivity of the cervical smear test. Furthermore the immunoenhanced Pap test is a method that
has the potential for automation. Large-scale clinical trials will be required to fully evaluate this approach, and these trials will require mAbs for full reproducibility and standardization.

References

Figure 1(A, B). Immunohistochemical staining of sections of HSILs with antibodies against PCNA. In HSIL, anti-PCNA antibodies show focal staining of nuclei in the surface layers (A: upper section); whereas anti-Mcm5 antibodies stain virtually all nuclei, including those at the epithelial surface. (B: lower section) (magnification ×250).

Figure 2. Immunohistochemical staining of cells obtained by cervical smear examination from surface of HSIL with antibodies against Ki-67 showing nuclear staining of the abnormal cells in the exfoliated sheets. (Magnification: ×250).
Figure 3. Staining of HSIL cells in cervical smears using a combination of immunohistochemical and Pap staining. The field contains metaplastic type HSIL cells. Superimposition of immunohistochemical method on the Pap smear still allows detailed examination of the cellular and nuclear morphology of positively immunostained cells (magnification: ×500).

Table 1. Percentage of immunohistochemical stained cells in the most superficial layers in sections of HSIL and LSIL cervix.

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<th>HSIL</th>
<th>LSIL</th>
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<tr>
<td>Mcm5</td>
<td>95</td>
<td>53</td>
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<tr>
<td>Cdc6</td>
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<td>43</td>
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<tr>
<td>PCNA</td>
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<td>Ki-67</td>
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<tr>
<th>Propotion &amp; markers</th>
<th>HSIL</th>
<th>LSIL</th>
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<tbody>
<tr>
<td>Median</td>
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<tr>
<td>Range</td>
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<td>78–100</td>
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Table 2. Comparison of the immunohistochemical Pap test with the conventional pathological diagnosis

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<tr>
<th>Classification by Anti-Mcm5 test</th>
<th>Classification by conventional criteria</th>
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<tr>
<td>Normal</td>
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