Role of Ki67 Protein in Pap Stained Cervical Cytology Smear in Differentiating Dysplastic Lesions from Benign Lesions of the Cervix

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Abstract

Purpose: The PAP smear has significantly reduce the mortality and morbidity of cancer cervix however there is some debates of it as interobserver variability and high false negative and false-positive rates so parameters of cell proliferation have emerged as important diagnostic and prognostic tools.

Aim of the Work: This present study aimed to evaluate the efficiency of Ki67 in differentiating dysplastic lesion from benign lesion of cervical cytology smear.

Patients and Methods: This cross-sectional study included 67 cases of cervicovaginal cytology smear of all gynecological cases in Benha University Hospital and Early Cancer Detection Unit, Pathology Department, Benha Faculty of Medicine during the period of October 2014-May 2015. Immunocytochemical Ki67 expression was both graded as well as Labelling Index was calculated. Statistical evaluation was carried out using the Fisher’s Extract test, Student t-test (p<0.05), Mann Whitney U test and Rho test, ROC curve, sensitivity, specificity and accuracy was calculated by using area under the curve.

Results: There was increase in mean Labelling Index of Ki67 immunocytochemical staining with increasing grade of dysplasia as the p-value among these groups was highly statistically significant (p-value <0.001). Labelling index was maximum in HSIL and minimum in benign lesions.

Conclusion: Ki-67 immunocytochemistry may be of value as a complementary tool in PAP stained cervical cytology and reducing the need of tissue biopsy as it is simple, reliable and easily applicable in routine cytosmears. Ki67 biomarker can be used in the evaluation of the proliferative activity and progressive potential of dysplastic and neoplastic changes.

Key Words: Immunocytochemistry – Cell proliferation markers – Ki67 – CIN/SIL – Cervical Pap smear.

Introduction

CERVICAL cancers are the second most frequent type of female cancer, responsible for about 5% of cancer deaths in females worldwide [1].

In Egypt, according to National Cancer Institute, Cairo University, provide widely varying estimates on the prevalence of pre-invasive cervical lesions ranging from 1% to 8%. Invasive lesions represent only 1.5% of all female invasive cervical lesions [2]. A monograph from the Egyptian National Cancer Registry of seven cancer centers of the Ministry of Health and Population, reported an incidence rate for all ages ranging from 0.12% to 0.77% depending on geographical regions, being more prevalent in Lower Egypt [3].

Papanicolaou (PAP) smear screening programs and histologic interpretation of biopsy specimen by the pathologist have significantly reduced the morbidity and mortality of cervical cancers. However, the great drawback of the PAP test is the high rate of false-negative and a false-positive result, the PAP test is not very accurate due to subjective test criteria. This limits the present screening programs and emphasizes the need for the identification of specific biomarkers for dysplastic epithelial cells to aid in primary screening and lesion diagnosis [4].

The expression level of Ki-67 indicated the status of cell proliferation. Ki67 is a non-histone protein located at nucleus, with a short half-life and it is known to be associated with cell proliferation, expressed in cells in the G1, S, G2 and M phases of the cell cycle, but it is absent in resting cells (G0 phase). In normal cervical squamous mucosa, Ki-67 is detected essentially in parabasal epithelial layers, the main source for cell renewal [13]. Some studies have showed that Ki-67 protein could be a biomarker in the evaluation of the proliferative activity and progressive potential of normal, dysplastic and neoplastic changes [5-7]. Therefore, the current study is carried to assess the possibility to use proliferative markers as
diagnostic immunocytochemical marker adjacent to PAP stained cervical smears.

**Patients and Methods**

This cross-sectional study was carried upon 67 cases of cervicovaginal cytology smear of all gynecological cases in Benha University Hospital and Early Cancer Detection Unit, Pathology Department, Benha Faculty of Medicine during the period of October 2014 – May 2015. The clinical data: (patient age, social level, age of marriage, smoking habits; (active smoking and passive smoking), parity, methods of contraception, complain, clinical examination finding); were collected for all the patients.

Cervical cells were collected using the AYER’S spatula and placed immediately in 95% ethyl alcohol. All smears were routinely stained with PAP stain. The cytological results were classified according to the Bethesda system (2001), using the following classes: Negative for Intraepithelial Lesion or Malignancy (NILM), Atypical Squamous Cells of Undetermined Significance (ASCUS), Low grade Squamous Intraepithelial Lesions (LSIL), High grade Squamous Intraepithelial Lesions (HSIL).

**Immunocytochemical staining:**

1. According to manufacturer instructions: Cervicovaginal cytology smear was obtained on positively charged slides (Fisher, USA).
2. The slides were put in plastic jar containing 100% alcohol for 10 minutes.
3. Slides were put in a slide rack in hot oven at temperature 70-75 for 15 minutes for proper fixation.
4. For antigen retrieval [10]:
   A) Slides were put in solution of TARGET RETRIEVAL [HIGH PH (50x)] (DAKO, Envision TMFLEX, USA).
   B) Slides were placed in microwave at temperature (650 WATT) for 20 minutes then at temperature (990 WATT) for another 20 minutes then back to (650 WATT) for 20 minutes.
   C) The slides were removed from the microwave and allowed to cool for 20 minutes at room temperature.
   D) Slides were then washed with distilled water for 15 minutes.
5. Blocking endogenous peroxidase activity was done by immersing the slides in 3% hydrogen peroxide in 30% methanol (DAKO, SM801, USA) for 10 minutes. Then smears were washed with Phosphate Buffered Saline (PBS) then distilled water to stop peroxidase activity.
6. One to two drops of the primary monoclonal antibody, Ki67 (DAKO, USA) pre-diluted ready to use; were applied to each smear. Slides were incubated in humid chamber for one hour at room temperature.
7. Slides were then rinsed with PBS then distilled water, then after blotting of excess water, each slide was incubated for 20 minutes with biotinylated secondary solution, then smears were rinsed PBS then with distilled water.
8. Slides were incubated for 20 minutes with streptavidin solution, and then smears were rinsed with PBS then distilled water.
9. Freshly prepared chromogen diaminobenzene (DAB) was used; it was incubated with slides for 3-5 minutes then washed with distilled water.
10. Slides were counter stained for 3 minutes with Mayer’s hematoxylin then washed and covered with mounting media (DPX).

Cervical cytology smear of 3 cases squamous cell carcinoma was used as positive controls. Neutrophils and granulocytes were used as internal positive control for Ki-67. For negative control, 1% nonimmune serum was used in place of primary antibody; the rest of the steps are the same. Cells were considered immunopositive if the nucleus showed homogeneous or punctate brown staining. The staining intensity was not graded to avoid subjective interpretation. Cytoplasmic staining without nuclear staining was not considered to be positive.

**Calculation of MIB-1 Labelling Index:**

Ki 67 labelling index (LI) was calculated by the number of positive cell sper 100 cervical epithelial cells in different areas under X400 magnification and the mean was calculated. Positive nuclei were expressed as the percentage of total nuclei counted. Ki67 labelling index was calculated as follows:

\[
\text{Labeling Index} = \frac{\text{No. of cells showing positive staining}}{\text{Total No. of cell}} \times 100
\]

**Grading of Ki-67 expression:**

The smears stained for Ki-67 proliferation (revealed as nuclear staining) were evaluated using scores from 1 to 3:
1: “+++” - High proliferation->50% positive cells.
2: “+” - Moderate proliferation-30%-50% positive cells.
3: “+” - Low proliferation-10-30% positive cells [13].

Statistical analysis:

The collected data were tabulated and analyzed using SPSS version 16 software (Spss Inc., Chicago, ILL Company). Categorical data were presented as number and percentages while quantitative data were expressed as mean and standard deviation. Fisher’s Exact Test (FET), ANOVA and Spearman’s correlation coefficient (rho) were used as tests of significance. ROC curve was used to determine cutoff value of Ki67 with optimum sensitivity and specificity in prediction of different cervical lesions. The accepted level of significance in this work was stated at 0.05 \( (p<0.05 \text{ was considered significant}) \). Quantitative variables will be presented as \( X \pm SD \) using ST (t) (standard t-test), Maun Whitney U test. ROC curve to detect cut off value of ki67 in detection of pre-invasive disease of cancer cervix with optimum sensitivity and specificity.

Results

Cytological diagnosis:

Cervicovaginal cytology smear diagnosed according to the Bethesda system (2001) to 4 categories: NILM category: 37 cases (55.2%) including squamous metaplasia, inflammatory smears. ASCUS category: 14 cases (20.9%). LSIL category: 10 cases (14.9%). HSIL category: 6 cases (9.0%). Ki-67 immuno-reactive cells showed dark brown, homogeneous or punctate staining, limited exclusively to the nucleus.

PAP smear examination of the cases revealed significant positive statistical correlations between PAP smear results and complains of patients and clinical examination findings. However, no significant statistical correlations were found between PAP smear results and age groups, age of marriage, social standard, smoking habits, parity nor methods of contraception.

Immunocytochemical diagnosis:

Immunocytochemical staining of Ki67 on cervicovaginal cytology smears revealed 51 cases (76.1%) with low proliferative index, 8 (11.9%) with inter mediate proliferative index and 8 cases (11.9%) with high proliferative index. There was a significant difference in Ki-67 immuno reactivity between the group containing normal or LSIL cases and the group containing HSIL \( (p<0.001) \) (Table 1).

Table (1): Correlation between Ki67 expression and PAP smear finding.

<table>
<thead>
<tr>
<th>Ki67</th>
<th>Low (10&lt;30%) (+)</th>
<th>Intermediate (30-50%) (+++)</th>
<th>High (&gt;50%) (++++)</th>
<th>Total Count</th>
<th>PAP Smear</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Count</td>
<td>% within PAP Smear</td>
<td>Count</td>
<td>% within PAP Smear</td>
<td>Count % within PAP Smear</td>
</tr>
<tr>
<td>NILM</td>
<td>37</td>
<td>100.0%</td>
<td>0</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>ASCUS</td>
<td>14</td>
<td>100.0%</td>
<td>8</td>
<td>80.0%</td>
<td>0%</td>
</tr>
<tr>
<td>LSIL</td>
<td>0</td>
<td>0.0%</td>
<td>2</td>
<td>20.0%</td>
<td>6%</td>
</tr>
<tr>
<td>HSIL</td>
<td>0</td>
<td>0.0%</td>
<td>6</td>
<td>100.0%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Fisher’s exact test =70.9.
NILM: Negative for intraepithelial lesion or malignancy. ASCUS: Atypical squamous cells of undetermined significance. LSIL: Low grade squamous intraepithelial lesion. HSIL: High grade squamous intraepithelial lesion. \( p \): \( p \)-value.

A linear progression of mean proliferative indices of Ki67 from normal to dysplastic lesion was found and the difference among these groups was statistically significant \( (p\text{-value}<0.001) \). The mean value of Labelling Index (LI) was found to increase as the nature of the lesion changed from Negative for Intraepithelial Lesions or Malignancy (NILM) \( (15.2\pm4.88) \) to High grade Squamous Intraepithelial Lesion (HSIL) \( (85.1 \pm 11.7) \) (Graph 1).

Graph (1): Correlation between Ki67 labelling index and PAP smear findings.
Role of Ki67 Protein in Pap Stained Cervical Cytology Smear

Fig. (1): These photomicrographs provide a morphologic comparison of various cervical lesions on Papanicolaou (PAP) smears, immunostaining of Ki-67, on Pap smear. The Pap smear demonstrates nuclear enlargement and hyperchromasia of atypical squamous cells in (A) Atypical Squamous Cell of Undetermined Significance (ASCUS) (PAP X400); (B) Low-grade Squamous Intraepithelial Lesion (LSIL) (PAP X400); (C) A hyperchromatic, crowded group of cells in High-grade Squamous Intraepithelial Lesion (HSIL) (PAP X400); immunodetection of Ki-67 demonstrated low proliferative labelling index staining for ASCUS (ABC X400) (D), and moderate proliferative labelling index staining for LSIL (ABC X400) (E), High proliferative labelling index staining for HSIL (F) (ABC X400).

LSIL : Low-grade Squamous Intraepithelial Lesion.
ASCUS : Atypical Squamous Cell of Undetermined Significance.
HSIL : High-grade Squamous Intraepithelial Lesions.

Diagnostic accuracy:

The diagnostic accuracy of Ki67 immunocytochemistry was determined by using ROC plots, these plots show the specificity (true negative fraction) and sensitivity (true positive fraction) of the test for all possible thresholds. The accuracy of the test is given by the area under the curve (AUC). Performance of Ki67 in Pap smear finding is shown in (Table 2).

Using ROC curve; immunocytochemical staining by Ki67 has 100% sensitivity, 98.4% specificity with 0.89 area under the curve in high grade squamous intraepithelial lesions and 100% sensitivity, 89.5% specificity with 0.90 area under the curve. However, Ki67 immunocytochemical staining has 78.6% sensitivity, 69.8% specificity with 0.67 area under the curve in diagnosis of atypical squamous cells of undetermined significance (ASCUS).

No significant correlation was found between labeling indices of Ki67s in relation to age, age of marriage, smoking habits, methods of contraception, parity, complains and clinical examination finding (Table 3).

Table (2): Ki-67 immunocytochemistry test performance as indicated by ROC plot.

<table>
<thead>
<tr>
<th></th>
<th>Cut off value</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
<th>NPV %</th>
<th>PPV %</th>
<th>Accuracy %</th>
<th>AUC</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NILM</td>
<td>&lt;20.5</td>
<td>81.1</td>
<td>76.7</td>
<td>81.1</td>
<td>76.7</td>
<td>89.4</td>
<td>0.89</td>
<td>0.81-0.97</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ASCUS</td>
<td>&gt;20.5</td>
<td>78.6</td>
<td>69.8</td>
<td>40.7</td>
<td>92.5</td>
<td>67.2</td>
<td>0.67</td>
<td>0.54-0.81</td>
<td>0.049</td>
</tr>
<tr>
<td>LSIL</td>
<td>&gt;30.5</td>
<td>100</td>
<td>89.3</td>
<td>62.5</td>
<td>100</td>
<td>89.6</td>
<td>0.90</td>
<td>0.82-0.98</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HSIL</td>
<td>&gt;62</td>
<td>100</td>
<td>98.4</td>
<td>100</td>
<td>85.7</td>
<td>99.7</td>
<td>0.997</td>
<td>0.98-1.0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

AUC : Area under the curve.
ASCUS : Atypical squamous cells of undetermined significance.
CI : Confidence interval.
HSIL : High grade squamous intraepithelial lesion.
LSIL : Low grade squamous intraepithelial lesion.
ROC : Receiver operating characteristic.
Table (3): Correlations between Ki67 expression and other Clinico-pathological parameters.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Ki67</th>
<th>Low (N=)</th>
<th>Intermediate (N=)</th>
<th>High (N=)</th>
<th>Fisher’s test</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
<td></td>
<td></td>
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<tr>
<td>PAP smear results</td>
<td>NILM</td>
<td>37 100</td>
<td>0 0</td>
<td>0 0</td>
<td>FET=70.9</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>ASCUS</td>
<td>14 100</td>
<td>0 0</td>
<td>0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LSIL</td>
<td>0 0</td>
<td>8 80</td>
<td>2 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HSIL</td>
<td>0 0</td>
<td>0 0</td>
<td>6 100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>20-30</td>
<td>13 25.5</td>
<td>2 25.5</td>
<td>1 12.5</td>
<td>FET=6.2</td>
<td>p=0.34</td>
</tr>
<tr>
<td></td>
<td>31-40</td>
<td>41.2 2</td>
<td>2 25.0</td>
<td>1 12.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>41-50</td>
<td>14 27.5</td>
<td>3 37.5</td>
<td>5 62.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>51-60</td>
<td>3 5.9</td>
<td>1 12.5</td>
<td>1 12.5</td>
<td></td>
<td></td>
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<tr>
<td>Standard</td>
<td>Low</td>
<td>14 27.5</td>
<td>1 12.5</td>
<td>3 37.5</td>
<td>FET=1.85</td>
<td>p=0.48</td>
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<tr>
<td></td>
<td>Medium</td>
<td>37 72.5</td>
<td>7 87.5</td>
<td>5 62.5</td>
<td></td>
<td></td>
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<tr>
<td>Smoking</td>
<td>No</td>
<td>50 98.0</td>
<td>7 87.5</td>
<td>8 100</td>
<td>FET=2.9</td>
<td>p=0.42</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>1 2.0</td>
<td>1 12.5</td>
<td>0 0</td>
<td></td>
<td></td>
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<tr>
<td>Smoking husband</td>
<td>No</td>
<td>5 9.8</td>
<td>1 12.5</td>
<td>1 12.5</td>
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</tr>
<tr>
<td></td>
<td>Yes</td>
<td>46 90.2</td>
<td>7 87.5</td>
<td>7 87.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parity</td>
<td>Nulliparous</td>
<td>10 19.6</td>
<td>1 12.5</td>
<td>0 0</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Low parity</td>
<td>41 80.4</td>
<td>6 75.0</td>
<td>7 87.5</td>
<td>FET=7.6</td>
<td>p=0.07</td>
</tr>
<tr>
<td></td>
<td>High parity</td>
<td>0 0</td>
<td>1 12.5</td>
<td>1 12.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method of contraception</td>
<td>No</td>
<td>13 25.5</td>
<td>2 25.0</td>
<td>0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hormonal</td>
<td>30 58.80</td>
<td>3 37.50</td>
<td>5 62.50</td>
<td>FET=5.6</td>
<td>p=0.19</td>
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<tr>
<td></td>
<td>Non hormonal</td>
<td>8 15.70</td>
<td>3 37.50</td>
<td>3 37.50</td>
<td></td>
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<td>Complain</td>
<td>Infection</td>
<td>32 62.70</td>
<td>6 75.00</td>
<td>5 62.50</td>
<td>FET=2.53</td>
<td>p=0.63</td>
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<tr>
<td></td>
<td>Infertility</td>
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<td>0 0</td>
<td>0 0</td>
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<td></td>
<td>Post coital bleeding</td>
<td>11 21.60</td>
<td>2 25.00</td>
<td>3 37.50</td>
<td></td>
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<tr>
<td>Clinical examination</td>
<td>Cervicitis</td>
<td>40 78.4</td>
<td>6 75</td>
<td>5 62.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cervical erosion</td>
<td>7 13.70</td>
<td>1 12.50</td>
<td>3 37.50</td>
<td>FET=5.1</td>
<td>p=0.53</td>
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<tr>
<td></td>
<td>Hypertrophied cervix</td>
<td>3 5.9</td>
<td>1 12.5</td>
<td>0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polyp</td>
<td>1 2.0</td>
<td>0 0</td>
<td>0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of marriage</td>
<td>Single</td>
<td>48 94.1</td>
<td>6 75.0</td>
<td>8 100</td>
<td>FET=3.39</td>
<td>p=0.2</td>
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<tr>
<td></td>
<td>Multiple</td>
<td>3 5.9</td>
<td>2 25.0</td>
<td>0 0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NILM: Negative for intraepithelial lesion or malignancy. ASCUS: Atypical squamous cells of undetermined significance. LSIL: Low grade squamous intraepithelial lesion. HSIL: High grade squamous intraepithelial lesion. FET: Fisher’s extract test. HS: High significance.

Discussion

Cervical cancer is the second most common cancer in women worldwide and remains a major cause of morbidity and mortality. The evolution of screening for cervical cancer has been considerable. Important efforts have been made to improve screening with introduction and combination of new methods and techniques [1].

In Egypt, according to the World Health Organization (WHO) most recent estimates, every year 866 women are diagnosed with cervical cancer and 373 die from the disease. Cervical cancer ranks as the 13th most frequent cancer among women in Egypt and the 10th most frequent cancer among women between 15 and 44 years of age. Data from Egyptian studies provide estimates on the prevalence of high-grade preinvasive cervical lesions ranging from 0.3% to 0.5% [3,14].

Papanicolaou (PAP) smear screening programs and histologic interpretation of biopsy specimen by the pathologist have significantly reduced the morbidity and mortality of cervical cancers. However, the great drawback of the PAP test is the high rate of false-negative and a false-positive result, the PAP test is not very accurate due to subjective test criteria. This limits the present screening programs and emphasizes the need for the identification of specific biomarkers for dysplastic epithelial cells to aid in primary screening and lesion diagnosis [4].

The present cross sectional study included 67 cases of cervico-vaginal cytology smears attending Gynecology and Obstetrics Department, Benha University Hospital and Early Cancer Detection Unit, Pathology Department, Benha Faculty of Medicine during the period of October 2014 – May 2015.
55.2% of cases diagnosed as Negative for Intraepithelial Lesions or Malignancy (NILM), 20.9% of cases diagnosed as Atypical Squamous Cell of Undetermined Significance (ASCUS), 14.9% of cases diagnosed as Low grade Squamous Intraepithelial Lesion (LSIL), 9.0% of cases diagnosed as High grade Squamous Intraepithelial Lesion (HSIL) according to the Bethesda System (2001).

In the present study, there was statistically high significant correlation between complains of patient and PAP smear results (p-value <0.001).

This result was in agreement with a study carried by Hippisley-Cox and Coupland., [15] who found that post-coital bleeding was associated with a 23-fold increased risk of cervical cancer despite adjustment for other risk factors and symptoms.

In contrast with our study Fakhrijou et al., [16] found that there is no significant correlation between background of genital infection and PAP smear findings. This contrasting finding may be due to different number of patients.

In the current study, no statistically significant correlation was found between social standard and PAP smear results (p-value=0.22). In contrast with Refaat et al., [17] and Singh et al., [18] who found that there was statistically significant correlation between social standard and PAP smear results. This contrasting result may be due to most of patient attending Benha University Hospital were of the same social level.

In this study, no statistically significant correlation was found between smoking habits of patient (active and passive smoking) and cervical intraepithelial neoplasia (p-value=0.19 and p-value=0.42) respectively. In contrast Rajkumar et al., [19] and Samir et al., [20] found statistically significant positive correlation between the smoking habits (active smoking) and cervical intraepithelial neoplasia. The explanation of these contrasting results may be due to that active smoking is not common among female at this social level. If smoking women, the duration of smoking, number of cigarettes smoked per day and adjustment of other factors as HPV infection and marital status, income, and access to health care should be taken in consideration.

In contrasting with the current study results Ward et al., [21] found that passive smoking has statistically significant correlation with abnormal PAP smear finding. These contrasting result may be explained as most of husbands works all the day mainly outdoors so passive smoker is not effective in this social level.

Our results showed no statistically significant correlation was found between parity and PAP smear results (p-value=0.22). This was in accordance with González et al., [22] as 80.6% of patient in the current study were with low parity according to the classification of Sikder et al., [23].

The present work found no significant correlation between methods of contraception and PAP smear results (p-value=0.4). In contrast to study carried out by De Villiers et al., [24] and Gadducci et al., [25] in which they found a significant positive correlation between the use of hormonal contraception and PAP smear results.

These contrasting results are explained by the difference in duration of use of hormonal contraception. Another explanatory mechanism is the association between HPV infection and the use of hormonal contraception as there is an increased risk for long-term hormonal contraception using with HPV-infected women and no evidence exists for an increase in cervical cancer among HPV-negative hormonal contraception users.

In the current study, no statistically significant correlation was found between age of marriage (early sexual habits) and PAP smear finding (p-value=0.51). In contrast of the study carried by Roy et al., [26] which found positive statistically significant correlation between early age of marriage and development of cervical intraepithelial neoplasia and carcinoma. It is explained by difference in religion, some religious practices, social and sexual habits.

Another explanation is that early first intercourse is a marker of high-risk behavior for HPV exposure as younger women are more susceptible to HPV infection.

In the current study, no statistically significant correlation was found between number of marriage (multiple sexual partners) and PAP smear finding (p-value=0.15). In contrast of the study carried by International Collaboration of Epidemiological Studies of Cervical Cancer., [27] which found positive statistically significant correlation between number of sexual partners and development of cervical intraepithelial neoplasia and carcinoma. It is explained by increased risk of HPV infection with increased number of sexual partners of patient herself and number of sexual partners to the husband. But this factor is limited in our community due to religious and social habits.
Concerning immunocytochemical study, Ki-67 is detected in the nucleus of proliferating cells in all active phases of the cell division cycle, but is absent in non-proliferating cells indicating that the Ki-67 antigen could be used as a marker for cells of the growth fraction.

According to Immunocytochemical results, Ki67 was detected as brown color positive staining confined to the nucleus with no cytoplasmic staining.

According to the current study, it was found that all thirty seven cases of Negative for Intraepithelial Lesions or Malignancy (NILM) was scored (+) having low proliferation with Labelling Index (LI) 10-30%. All Fourteen cases of Atypical Squamous Cell of Undetermined Significance (ASCUS) was scored (+) having low proliferation with labelling index 10-30%. On the other side out of ten cases diagnosed as Low grade Squamous Intraepithelial Lesion (LSIL), two cases have high proliferation with labelling index >50% (+++), while eight cases showing moderate proliferation with labelling index 30-50% (++). All six cases of High grade Squamous Intraepithelial Lesion (HSIL) have high proliferation with labelling index >50% (+++).

In the current study, it is found alinear progression of mean proliferative indices of Ki67 from normal to dysplastic lesion and the difference among these groups was statistically significant \(p\)-value < 0.001. The mean value of Labelling Index (LI) was found to increase as the nature of the lesion changed from Negative for Intraepithelial Lesions or Malignancy (NILM) (15.2 ± 4.88) to High grade Squamous Intraepithelial Lesion (HSIL) (85.1 ± 11.7). Similar results were observed by Anju & Mati [11]; Munhoz et al., [28] and Gupta et al., [12].

Bulten et al., [29] by similar study analyzed cervical biopsy and Pap smear in terms of Ki-67 presentation and activity. This study showed that percentage of Ki-67 expression in Cervical Intraepithelial Neoplasia (CIN) samples was significantly higher than normal samples so it can distinguish between neoplastic or preneoplastic samples and non-neoplastic samples completely, in agreement with our study.

Also the mean Labelling Index (LI) in cervical dysplasia increased with increasing grade of dysplasia with mean LI in Atypical Squamous Cell of Undetermined Significance (ASCUS) was (21.2 ± 6.19), Low grade Squamous Intraepithelial Lesion (LSIL) (44.3 ± 11.85) and High grade Squamous Intraepithelial Lesion (HSIL) (85.1 ± 11.7). Similar results were obtained by Conesa-Zamora et al., [30] and Simionescu et al., [31] who also showed an increase in mean basal Labelling as moving from Atypical Squamous Cell of Undetermined significance (ASCUS) to High grade Squamous Intraepithelial Lesion (HSIL).

In agree with Sahebali et al., [32] and Goel et al., [33] statistical analysis of our results showed a significant difference of mean Labelling Index between high grade squamous intraepithelial lesion (HSIL) groups (85.1 ± 11.7) which still twice that of Low grade Squamous Intraepithelial Lesion (LSIL) (44.3 ± 11.85), indicating that these marker may be helpful in diagnosing and differentiating squamous intraepithelial lesions.

In the current study, it was observed that high Ki67 labelling index was indicative of high-grade lesion but no cases of High grade Squamous Intraepithelial Lesion (HSIL) lesion with low proliferative index was detected. On the contrary out of ten cases diagnosed as Low grade Squamous Intraepithelial Lesion (LSIL), we had two cases of Low grade Squamous Intraepithelial Lesion (LSIL) smears (confirmed as Cervical Intraepithelial Neoplasia grade I (CIN 1) on histology) that had high proliferative indices on immunocytochemistry staining with Ki67.

This could be explained by the fact that there were more HPV-16 infections in the High grade Squamous Intraepithelial Lesion (HSIL) group which had high proliferative index, so presence of highly proliferative index in Low grade Squamous Intraepithelial Lesion (LSIL) is highly suggestive to presence of HPV-16.

Low grade Squamous Intraepithelial Lesion (LSIL) mainly represents morphological correlates of active HPV replication (e.g. koilocytes), whereas high grade squamous intraepithelial lesion (HSIL) is characterized by morphological alterations indicative of transformation, primarily increasing nuclear alterations [34].

The overlap of the number of Ki-67 immunoreactive cells was relatively broad between the cytological groups. This overlap in immunopositive counts in the different groups could partly be explained by the fact that normal proliferating cells from the basal layer of the epithelium will regularly be seen as positive cells. Such (para-) basal cells can be recognised on the slides used for immunocytochemistry [32]. To overcome this misinterpretation, the diagnostic accuracy of Ki-67 immunocytochemistry was determined using ROC plots.
The diagnostic accuracy of the test is given by
the Area Under the Curve (AUC). In agreement
with Sahebali et al., [32] who found that the accuracy
for Atypical Squamous Cell of Undetermined Signi-
ficance (ASCUS) is good enough to be used as
an adjunct to PAP smear (sensitivity 68.0%, spec-
ificity 61.0%, PPV 87.1% NPV 32.6%), the current
study found that the performance of Ki67 in diag-
nosis of Atypical Squamous Cell of Undetermined
Significance (ASCUS) was (sensitivity 78.6%, speci-
ficity 89.5%, PPV 40.7%, NPV 92.5%, accuracy
67.2% with AUC of 0.67 and 95%CI of 0.54-
0.81).

Hypothetically, the cytological diagnosis of
Atypical Squamous Cell of Undetermined Signif-
icance (ASCUS) combined with a high count of
Ki-67 immunopositive cells could be useful to
identify those women who need follow-up, because
these would be cases where the cell cycle is dis-
rupted Gupta and Rajwanshi, [34]. The current
study found that all cases diagnosed as Atypical
Squamous Cell of Undetermined Significance
(ASCUS) by PAP smear had a low proliferative
index by Ki67 immunocytochemical stain, so these
cases did not need follow-up. In contrast of Gupta
and Rajwanshi [34].

Pirog et al., [35] and Ziemke et al., [36] in agree-
ment with the current study, found that the immu-
nohistochemical staining of Ki-67 leads to a signif-
icantly better Positive Predictive Value (PPV) and
a very good Negative Predictive Value (NPV)
sensitivity 100%, specificity 89.5%, PPV 62.5%,
NPV 100% Accuracy of 89.6%, with AUC of 0.90
and 95%CI of 0.82-0.9) for diagnosis of Low grade
Squamous Intraepithelial Lesion (LSIL).

In the current study, Ki67 has high sensitivity
and specificity in detection of High grade Squamous
Intraepithelial Lesion (HSIL) as sensitivity was
100%, specificity was 98.4% with Positive Predic-
tive Value (PPV) 85.7% and Negative Predictive
Value (NPV) 99.3%, 95% Confidence Interval (CI: 96.3-100% and 95%CI of 0.81).

In conclusion, the increasing of Ki-67 expres-
sion with increasing grade of dysplasia and increase
in progression from dysplasia to carcinoma shows
that it could be used as biomarker of dysplasia and increase
in progression from dysplasia to carcinoma shows
that it could be used as biomarker in the evaluation
of the proliferative activity and progressive poten-
tial of dysplastic and neoplastic changes. Ki-67
immunochemistry may be of value as a com-
plementary tool in PAP stained cervical cytology
for screening and reducing the need of tissue biopsy
as it is simple, reliable and easily applicable in
routine cytosmears.

Researches on large number of patient may be
needed to determine approperate cut off value to
avoid the overlap in numbers of immunopositive
cells between groups, identification of the ability
to perform double staining (PAP stain and Ki67
immunostaining) on the same slide for proper
domain and follow-up of cases to confirm diag-
nosis of dysplastic changes and exclude parabasal
cells immunostaining is recommended.

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Role of Ki67 Protein in Pap Stained Cervical Cytology Smear


