

## Histopathology of cervical lesions and their association with biomarker Ki-67

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### Abstract

**Introduction:** Histomorphological grading of cervical malignancy is important for clinical management. Ki-67, a proliferation marker known as predictive factor for tumor development, is a nuclear antigen expressed during all active phases of the cell cycle. In normal cervical squamous mucosa, Ki-67 is detected essentially in parabasal epithelial layers. Ki-67 immunohistochemistry positivity demonstrates the increasing proliferation in low and high grades of intraepithelial lesions.

**Objectives:** To analyse the expression of Ki-67 immunostains on nonneoplastic, dysplastic and neoplastic specimens and to study their role in increasing the diagnostic accuracy in equivocal cases on histopathology.

**Materials and Methods:** It was a cross sectional study done over a period of seven months after Institutional Ethics Committee permission. Histopathological examination and clinical diagnosis of all included patients was done. Immuno-histochemical staining for Ki-67 was performed and evaluated light-microscopically. A tumor was considered positive with significant proliferating activity only if nuclear Ki-67 accumulation was identified in at least 10% of all malignant cells in a tissue section.

**Observations:** Histopathological examination and clinical diagnosis shows cervical carcinoma was the commonest and was seen in 82.50% of patients. Followed by chronic cervicitis (7.5%), prolapse (5%) and Non-neoplastic cervical growth (5%). Ki-67 grading <10% (score 0) was seen in 5(6.25%) cases. 10-30% (score 1) was seen in 22(16.25%) cases. 30-50% (score 2) was seen in 13(16.25%) cases and >50% (score 3) was seen in 40(50.0%) of cervical biopsies. Ki-67 immunostaining was negative in chronic cervicitis cases.

**Conclusion:** Histological diagnosis of cervical biopsy samples, is observed to have significant inter-observer discrepancies. Therefore, there is a need for additional sensitive and specific biomarkers to improve cervical cancer screening which can improve standardization and quality control of histopathological diagnosis.

**Keywords:** Neoplastic and non-neoplastic cervical lesions, Histopathological examination, Ki-67 grading.

### Introduction

Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of death due to cancer in females worldwide.<sup>1</sup> Despite the implementation of Papanicolaou test (PAP test) that has successfully brought dramatic reduction in the incidence and mortality worldwide caused by cervical cancer, there are still a substantial amount cervical cancers occurring in women who are adequately screened, proving diagnostic limitation of the Pap test.<sup>1</sup>

Epidemiological and molecular studies have shown that human papilloma virus (HPV) is the most important etiological agent for cervical cancer.<sup>2</sup> HPV infection of the cervix is transmitted venereally, and it has a predilection for the metaplastic squamous epithelium. It may remain dormant for long periods or become productive, with release of infectious virus in the terminally differentiated squamous epithelium. HPV-type specific oncoproteins E6 and E7 bind to cellular regulatory proteins, specifically, the p53 tumor suppressor protein and the retinoblastoma protein (Rb). Inactivation of these factors, either by degradation (p53) or functional inactivation (Rb), leads to disruption of the cell cycle and increased proliferation, thought to ultimately give rise to carcinoma.<sup>2</sup>

Ki-67, a proliferation marker known as predictive factor for tumor development, is defined as a nuclear antigen (associated with hetero- and euchromatin) expressed during all active phases of the cell cycle (G1, S, G2, M) except G0. The level of Ki-67 expression is used to determine the cell

proliferation status.<sup>3</sup> Ki-67 is present only in dividing cells, either normal or tumor, but absent in resting cells, only cells that over-express p53 or p21 may be assessed by using Ki-67. In normal cervical squamous mucosa, Ki-67 is detected essentially in parabasal epithelial layers (the main source for cell renewal), but also in certain basal layers.<sup>4</sup> Ki-67 immunohistochemistry positivity demonstrates the increasing proliferation in low and high grades of intraepithelial lesions. Histomorphological grading of cervical malignancy is important for clinical management. Hence the current study is undertaken to assess the utility of potent biomarker Ki -67 in evaluating cervical biopsies in diagnosing and grading of cervical malignancies.

In this study, immunohistochemical staining of formalin fixed paraffin embedded samples of cervical biopsy was done with an aim to analyze the expression of Ki-67 immunostains on nonneoplastic, dysplastic and neoplastic specimens and to study their role in increasing the diagnostic accuracy in equivocal cases on histopathology.

### Materials and Methods

This was a cross sectional study conducted by the Department of pathology in a tertiary care teaching hospital of mid Gujarat, from June 2016 till December 2016 i.e. seven months of duration. Approval was obtained prior to the study from Institutional Ethical committee. Subjects were enrolled in the study based on inclusion and exclusion criteria.

### Inclusion and Exclusion Criteria

All the cervical biopsies submitted to Histopathology section of Pathology department were included in the study while known case of cervical carcinoma and Tissue sections with inadequate study material were excluded from the study.

The demographic details of the patient like age, significant family and personal history, history of other diseases were collected and recorded as per proforma.

### Assessment Tools

The sections stained for Ki-67 proliferation index (revealed as nuclear staining) are evaluated using scores from 1 to 3: “+” meaning low-proliferation, 10–30% positive cells; “++” moderate proliferation, 30–50% positive cells; “+++” meaning high-proliferation (more than 50% positive cells).<sup>5</sup>

### Method of Study

A sample of approximately 80 cervical biopsy specimens collected from histopathology section after obtaining ethical approval for use of all specimens. All samples were fixed in formalin and embedded in paraffin wax by conventional techniques and stained with Hematoxylin and eosin and the slides are studied and histopathological grading of cervical malignancy is done.

Grading of cervical lesions will be done based on the proportion of stained cells and the intensity of staining. Thereby, assessing the utility of immune marker in discriminating the cervical neoplastic lesions from non-neoplastic equivocal lesions of cervix.

Immunohistochemical staining was performed using Peroxidase anti peroxidase method (PAP) using paraffin embedded blocks cut into 3–4 µm thick sections. Biogenex reagents were used for the antigen retrieval and IHC staining process. Tris-EDTA based antigen retrieval solution with a pH 6 was used for Ki-67 staining. The heating cycles followed in the AG unit (Biogenex EZ-Retriever System v.2.1) were two cycles of 10 minutes and 5 minutes each at 95°C, with intermittent refilling of the antigen retrieval solution. AG unit works on the principle of application of heat energy for varying length of time on formalin-fixed, paraffin-embedded tissue sections immersed in ‘Antigen retrieval solution’ with a unique feature of temperature monitoring system.

Thereafter the slides were brought down to room temperature and taken through the steps of wash with PBS, peroxide block, power block and incubated with only one drop of primary antibodies on the tissue section: Ki-67 (clone BGX-Ki67, IgG1, Kappa class immunoglobulin with protein concentration of 10-15mg/ml) for 1 hour in humidity chamber at room temperature. After this, slides were again washed with PBS, and treated with super enhancer and polymer HRP and then again washed with PBS and then the secondary antibody to be exhibited, thereafter DAB chromogen was added. The slides were then washed with water, counterstained with Hematoxylin and blued. Then slides were serially dehydrated in alcohol, cleared in xylene and thereafter mounted using DPX. After drying, the test

slides were examined along with the control sections stained simultaneously.

Negative external control sections for each case were treated identically except that the primary antibody was replaced with phosphate buffer saline (PBS). Positive external control sections containing tissue from a tonsil (for Ki-67) was included in each staining run.

### Assessment of Ki-67

The Ki-67 immunostained sections were light-microscopically evaluated using a total magnification of 400x and 6-9 sites within the biopsy section are examined, excepting the areas with tissue enfolding, necrosis and haemorrhagic infiltrate. Nuclear staining was regarded as positive whereas cytoplasmic staining was considered as artefact. MIB-1 labelling index (LI) was determined by counting about 500 tumor cells, and was calculated as the percentage of positive labelled nuclei. A tumor was considered positive with significant proliferating activity only if nuclear Ki-67 accumulation was identified in at least 10% of all malignant cells in a tissue section.

### Statistical Method

The data are tabulated and frequencies and percentages are calculated for qualitative variables. Categorical variables were compared using Chi-square test and p value was calculated. p-value less than 0.05 was considered statistically significant.

### Results

Histomorphological study of premalignant and malignant lesions of the cervical biopsy was undertaken by the Department of Pathology over a period of six months. During this study period a total of 80 cervical biopsies were received and premalignant and malignant lesions of Cervix was studied.

Age distribution of all cases shows that highest number was in 41-50 years of age (37.50%) followed by 31-40 years of age (26.25%). The extremes of age had less incidence of cervical lesions. [Fig. 1] The clinical diagnosis showed that cervical carcinoma was the commonest and was seen in 82.50% of patients. Followed by chronic cervicitis (7.5%), prolapse (5%) and non-neoplastic cervical growth (5%). [Table 1] Age wise distribution of cervical carcinoma shows majority of patients were in 4th and 5th decade.

All the 64 malignant tumours were epithelial in origin. 1 case (1.54%) was microinvasive carcinoma and other 63 cases (98.7%) were invasive. Squamous cell carcinoma was diagnosed in majority of cases (93.75%), followed by 2 cases (3.13%) of adenocarcinoma, and 1 case (1.58%) of adenosquamous carcinoma and 1 case (1.54%) of rhabdomyosarcoma. [Table 2] Of the 61 cases of squamous cell carcinoma, 1 case (1.67%) was microinvasive squamous cell carcinoma, 54(88.33%) cases were large cell non keratinizing type (LCSCC), 5(8.33%) cases were keratinizing type and 1(1.67%) case was acantholytic squamous cell carcinoma. [Table 3]

Out of 60 cases of invasive squamous cell carcinoma 51 cases (85%) showed associated Cervical Intraepithelial Neoplasia (CIN) changes in the adjacent epithelium. [Table 4]

#### Ki-67 immunostaining and Assessment of Ki-67

The Ki-67 immunostained sections were light-microscopically evaluated using a total magnification of 400x and 6-9 sites within the tumour were examined, excepting the areas with tissue enfolding, necrosis and haemorrhagic infiltrate. Nuclear staining was regarded as positive whereas cytoplasmic staining was considered as artefact. Ki-67 labelling index (LI) was determined by counting about 500 tumor cells, under 400x magnification and was calculated as the percentage of positive labelled nuclei. A tumor was considered positive with significant proliferating activity only if nuclear Ki-67 accumulation was identified in at least 10% of all malignant cells in a

biopsy section. The results for Ki-67 were scored by a semi-quantitative scoring system as mentioned in Table-5.<sup>6</sup>

In present study Ki-67 grading <10% (score 0) was seen in 5(6.25%) cases. 10-30% (score 1) was seen in 22(16.25%) cases. 30-50% (score 2) was seen in 13(16.25%) cases and >50% (score 3) was seen in 40(50.0%) of cervical biopsies. [Table 6]

Table 7 shows Expression of Ki-67 in cervical tissues with different histopathological diagnosis. Ki-67 immunostaining was negative in chronic cervicitis cases. And it showed positive expression in hyperplastic squamous epithelium in the basal half of the epithelium. There was intense positive staining in full thickness of the epithelium of HSIL. Invasive carcinomas showed diffuse and strong expression of Ki-67 immunostaining in all cases of cervical biopsies.

**Table 1:** Clinical diagnosis of all cases of cervical lesions

Clinical Diagnosis	Number of cases	Percentage
Chronic Cervicitis	6	7.50%
Carcinoma Cervix	66	82.50%
Prolapse	4	5.00%
Non-neoplastic Cervical Growth	4	5.00%
Total	80	100.00%

**Table 2:** Type of invasive carcinoma

Tumour type	Number of cases	Percentage
Squamous Cell Carcinoma (SCC)	60	93.75%
Glandular	2	3.13%
Adenosquamous	1	1.58%
Rhabdomyosarcoma	1	1.54%
Total	64	100.00%

**Table 3:** Histologic types of Squamous Cell Carcinoma (SCC)

Tumour Type	Number of Cases	Percentage
Microinvasive SCC	1	1.67%
LCNKSCC	54	88.33%
Keratinizing SCC	5	8.33%
Acantholytic SCC	1	1.67%
Total	61	100%

**Table 4:** Associated CIN changes in subtypes of SCC

Tumour type	Associated CIN Changes		Total No. of Cases
	Present	Absent	
Microinvasive SCC	1	0	1
LCNKSCC	45	8	53
Keratinizing SCC	4	1	5
Acantholytic SCC	1	0	1
Total	51	9	60

**Table 5:** Ki-67 Scoring system

Staining	Score
<10%	0
10-30%	1
30-50%	2
>50%	3

Negative - 0, Positive - 1-3

**Table 6:** distribution of IHC biomarker Ki-67 grading

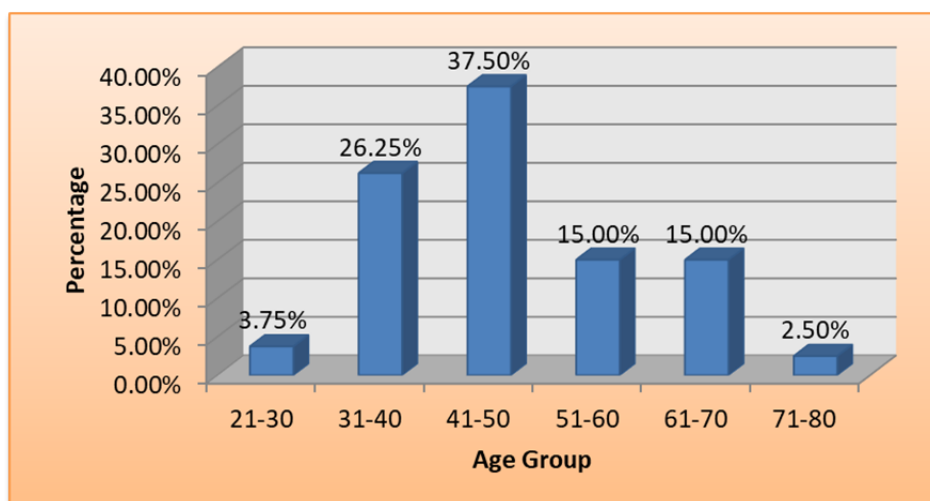
Ki-67 grading	Number of cases	Percentage
0	5	6.25%
1	22	27.50%
2	13	16.25%
3	40	50.00%
Total	80	100%

**Table 7:** Expression of Ki-67 in cervical tissues with different histopathological diagnosis

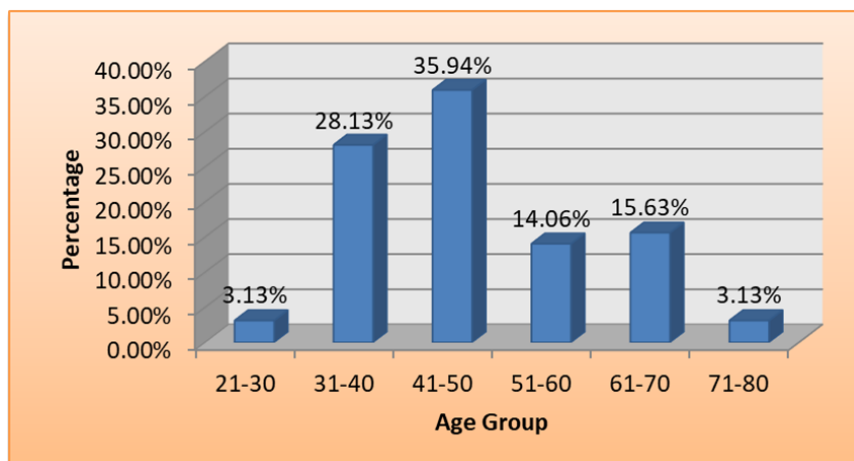
Histopathological diagnosis	Number of cases	Ki-67 score- immunostaining (%)			
		0-negative	1-positive	2-positive	3-positive
Hyperplastic squamous epithelium	4	0	2	1	1
HSIL	1	0	0	0	1
Chronic cervicitis	4	3	1	0	0
Adenosquamous SCC	1	0	0	1	0
Large cell non keratinising SCC	56	0	12	9	34
Keratinising SCC	5	0	2	2	1
Adenocarcinoma	2	0	1	0	1
Rhabdomyosarcoma	1	0	1	0	0
Others	7	2	3	0	2
Total	80	5	21	14	40

**Table 8:** Correlation between histological diagnosis and ki-67 immunostaining

Cervical Carcinoma	Ki-67		Total
	Negative	Positive	
Negative	5	11	16
Positive	0	64	64
Total	5	75	80



**Fig. 1:** Age distribution of all cases



**Fig. 2:** Age wise distribution of cervical carcinoma patients

### Discussion

Uterine cervical cancer is a major health problem, with a 75% decrease in the incidence over the past 50 years in developed countries, it is still the second most common cause of cancer related morbidity and mortality among women in developing countries.<sup>7</sup>

In this study malignant tumours constituted 64(80%) cases of the total 80 cases of cervical biopsies. This correlates with Solapurkar ML who reported an incidence of 1.08% cases of malignancy in her study.<sup>8</sup>

The incidence of cervical carcinoma is highly dependent on age.<sup>7</sup> Mean age at diagnosis of invasive carcinoma in the present study was 48.46 years. A similar mean age of 47 years has been observed for diagnosis in the United States. [7] In a study conducted in central India mean age at diagnosis was 46.20 years.<sup>9</sup>

Squamous cell carcinoma was the commonest diagnosis. It was diagnosed in 60(93.75%) cases, including 1(1.5%) case microinvasive carcinoma, 2(3.13%) cases of Adenocarcinoma, 1(1.58%) case of adenosquamous and 1(1.54%) case rhabdomyosarcoma. The incidence of different types of cervical malignancies in the present study is in accordance with other studies.<sup>7,9-12</sup>

Mean age of patients with invasive squamous cell carcinoma of uterine cervix in the present study was 48.0 years. Similar observations were made by different studies.<sup>7,13</sup> Out of 60 invasive squamous cell carcinomas studied, large cell non keratinizing type was the commonest seen in 54 cases (88.52%). Keratinizing type was seen in 5 cases (8.20%). Acantholytic squamous cell carcinoma was seen in 1 case (1.64%). Observation in this study regarding the predominant cell type of squamous cell carcinoma correlates with the study by Lowe D et al<sup>10</sup> who had also observed LC NK SCC as the commonest histological type of squamous cell carcinoma. Associated CIN was seen in 51 cases (85%) of a total of 60 invasive squamous cell carcinoma.

In the present study there was one case (1.6%) of non keratinizing squamous cell carcinoma following radiotherapy of the total invasive SCC. The histomorphological changes were similar to features of

partial pathological response described by Zannoni GF et al.<sup>14</sup> These authors had observed 48% of the cases with features of partial pathological response following radiochemotherapy in their study.<sup>14</sup>

### Ki-67 Immunostaining

The grade of dysplasia had been found to correlate well with the Ki-67 expression which is why it has been increasingly studied for the evaluation of lesions of the cervix.

The high specificity and reproducibility with this staining have led to its increasing utility in screening. Diffuse positive staining is found to be consistent with CIN, but Ki-67 staining cannot differentiate between dysplasia and immature squamous metaplasia. However, Ki-67 staining is advantageous over HPV testing as subclinical HPV infections show negative staining, and it is a simple, low-cost laboratory technique.

In present study total 64 cervical carcinomas were found out of 80 cervical biopsies. Correlation between histological diagnosis and ki-67 immunostaining seen in table 8. According to this correlation study sensitivity and specificity of Ki-67 staining was 85% and 95% with 94% PPV (Positive predictive value) and 11% NPV (Negative predictive value). The p value 0.001, which was considered significant.

### Conclusion

Histological diagnosis of cervical biopsy samples, is observed to have significant inter-observer discrepancies. Therefore, there is a need for additional sensitive and specific biomarkers to improve cervical cancer screening which can improve standardization and quality control of histopathological diagnosis.

**Conflict of Interest:** None.

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