Evaluation of Ki-67 Immunostaining in the Differential Diagnosis of Low Grade Squamous Intraepithelial Lesion and Normal Cervix

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Abstract

Objective: Ki-67 immunostaining is used as an adjunct in the differential diagnosis of squamous intraepithelial lesions (SILs) of the cervix. The aim of this study was to determine an index for positive Ki-67 staining and the Ki 67-positive cell clusters for distinguishing normal cervix and low-grade squamous intraepithelial lesions (low-grade SILs).
Materials and Methods: Thirty-four cervical specimens, in which 17 were previously diagnosed as normal and 17 low-grade SILs were included in the study. Ki-67 immunohistochemical staining was performed using avidin biotin complex.
Results: After reevaluating the cases by means of histo-pathological and Ki-67 immunostaining features, 21 of 34 cases were diagnosed as normal cervix and 13 were diagnosed as low-grade SILs. Ki-67 index was higher in low-grade SILs ($p<0.05$). Although Ki-67 positive cell clusters was not observed in normal cervix, 77% of 13 low-grade SILs had positive cell clusters ($p<0.05$).
Discussion: Ki-67 immunostaining may be considered as an easy and reproducible technique in differential diagnosis of normal cervix and low-grade SIL.

Keywords: cervix, Ki-67, immunohistochemistry, low-grade squamous intraepithelial lesion

Özet

Düşük Gradlı Skuamöz İntraepitelyal Lezyon ve Normal Serviksin Ayırıcı Tanısında Ki-67 İmmün Boyamanın Değerlendirilmesi

Amaç: Ki-67 immün boyama, serviksin skuamöz intraepitelyal lezyonlarında (SIL) ayırıcı tanıda kullanılan tanısal bir testtir. Bu çalışmanın amacı, düşük gradlı SIL ve normal serviksin ayırıcı tanısında Ki-67 indeksini ve Ki-67 pozitif hücre kümelerini belirlemektir.
Materyal ve Metot: Çalışmada, patolojik incelemede 17'si önceden normal serviks, diğer 17'si düşük gradlı SIL tanısı konmuş toplam 34 servikal örneğe Ki-67 immünohistokimyasal boyama yapıldı.
Sonuçlar: Örnekler, histopatolojik olarak ve Ki-67 immün boyama ile tekrar değerlendirdikten sonra 34 örneğin 21'ine normal serviks ve 13'üne düşük gradlı SIL tanısı kondu. Ki-67 indeksi, düşük gradlı SIL olgularında anlamlı olarak yüksek idi ($p<0.05$). Ki-67 pozitif hücre kümeleri normal servikste gözlenmedi, 13 düşük gradlı SIL olgusunun %77'sinde ise Ki-67 pozitif hücre kümeleri gözlendi ($p<0.05$).
Tartışma: Ki-67 immün boyama, normal serviks ve düşük gradlı SIL olgularının ayırıcı tanısında kolay ve kullanışlı bir teknik olarak düşünülebilir.

Anahtar sözcükler: serviks, Ki-67, immünohistokimya, düşük gradlı skuamöz intraepitelyal lezyon

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Introduction

Squamous intraepithelial lesion (SIL) of the cervix comprises a morphologically and biologically heterogeneous group of lesions that may be a precursor of invasive squamous cell carcinoma of the uterine cervix. Thus, it is crucial to make the diagnosis of SIL and to assess the grade of the lesion. The distinction of SIL from non-neoplastic lesions, however, can be difficult because those lesions may mimic SIL. Particularly, in some specimens low-grade SIL could not be determined easily. Not only distinguishing non-neoplastic lesions from high-grade SIL but also from normal cervix could be difficult. Additional methods are required to make the correct diagnosis and to identify of the grade of the lesion (1-8).

Previous studies have shown that monoclonal antibodies to the cell proliferation associated nuclear antigen Ki-67 are useful for objective, reliable classification of the neoplastic and non-neoplastic lesions in cervical epithelium (1,2,5,9,10). The staining patterns, the distribution of the positive staining nuclei and quantitative features could be variable. Studies have shown that Ki-67 proliferative activity is highly correlated with the changes in the cervical epithelium. Ki-67 nuclear staining is observed in normal mucosa only in parabasal and basal cells and its expression is seen in higher epithelial levels in SIL (1,2,5,9-11). Although several studies have suggested that Ki-67 evaluation can be valuable in the diagnosis of cervical lesions, the definitions of this association were not always clear. Therefore, there are different techniques to determine and count Ki-67 positive cells. Both quantitative Ki-67 features and positive Ki-67 positive cell clusters are found to be useful to analyze neoplastic versus normal cervical epithelium (1,2,4-8,10).

Our study is designed to investigate the Ki-67 index and Ki-67 positive cell clusters in distinguishing SIL from normal cervix. Our aim was to evaluate whether Ki-67 is helpful in suggestive but not diagnostic of low-grade SIL cases.

Materials and Methods

Totally 32 cervical biopsies, 17 of which were diagnosed as SIL and 17 as normal cervix by different pathologists, were selected from the archieves of the Department of Pathology in Cumhuriyet University Hospital between 2000 and 2005. Standard 4 μm thick, hematoxylin and eosin stained section of 34 cervical samples, were reevaluated and reclassified according to the Bethesda terminology (12). In addition, to confirm histopathologic diagnosis, all case samples were stained with Ki-67 using avidin-biotin complex method as previously described (1).

Immunohistochemistry

Formalin fixed paraffin embedded specimens were cut 4 μm sections. For each case 2 sections were obtained. Slides were deparaffinized in xylene and rehydrated in a series of ethanol. Incubating slides with 3% hydrogen peroxide for 10 minutes blocked endogenous peroxidase and nonspecific background staining. Non-enzymatic antigen retrieval was performed to improve staining results of formalin-fixed tissues; one of the sections was boiled in 10 mM citrate buffer pH6.0 and other sections were boiled with EDTA buffer (Cat no: AP-9004-500, Lab Vision). Then retreatment was done in a microwave for 15 minute. After washing with phosphate-buffered saline (PBS) for 5 minutes, slides were blocked with normal serum for 20 minutes, followed by incubation with the specific primary antibody for 60 minutes. Rabbit monoclonal antibody Ki-67, Clone SP6 was used as the primary antibody (Lab vision, cat no: RM-9106-R7). After rinsing with PBS for 5 minutes, sections were incubated with a biotinylated secondary antibody (Lab vision, TP-125-BN) for 20 minutes. After washing with PBS, slides were incubated with avidin-biotin complex (Lab vision, TS-125-HR) for 30 minutes. AEC chromogen (Lab vision, TA-060-HA) was applied. All slides were lightly counterstained with Mayer hematoxylin for 30 seconds before dehydration and mounting.

Sections of human tonsil were used as positive control, and a negative control without primary antibody was applied. The parabasal cells of the squamous epithelium served as an internal positive control. Quantitation of Ki-67 immunostaining was made by counting separately the positively and negatively stained nuclei in the lower third of the full thickness epithelium in the region of highest staining. At least two visual fields were counted per layer using a 40X objective with a 10X eye piece. For each case approximately 400 nuclei was counted. Only moderate (2+) to strong (3+) staining intensity was scored as positive. Ki-67 index was expressed as the percentage of positively stained nuclei to all epithelial nuclei counted in each layer (10).

Ki-67 staining features were also investigated according to Pirog et al. Ki-67 staining was defined as positive when a cluster of at least two strongly stained nearby epithelial nuclei was present in the upper two thirds of the epithelial thickness anywhere within the lesion (5).

Statistical Analyses

Data were presented as mean ±SD. The comparison of Ki-67 index in two groups was analyzed by Mann Whitney U test. The confidence interval for Ki-67 index was determined by one sample *t*-test. Ki-67 positive or negative cell cluster results were analyzed by χ^2 test (Chi square). The sensitivity and specificity of cell clusters were also calculated. A value of $p<0.05$ was considered as statistically significant.

Results

We reevaluated 34 cases, 17 of which had been previously diagnosed as low-grade SIL and 17 had been classified as normal cervix. After reevaluation by histopathologic and immunohistochemical investigation, 21 cases were diagnosed as normal cervix (Figure1) and 13 cases were classified as low-grade SIL (Figure 2). Table 1 and 2 summarize the diagnoses after reevaluation, Ki-67 index and positivity of Ki-67 cell clusters in low-grade SIL and normal cases, respectively.

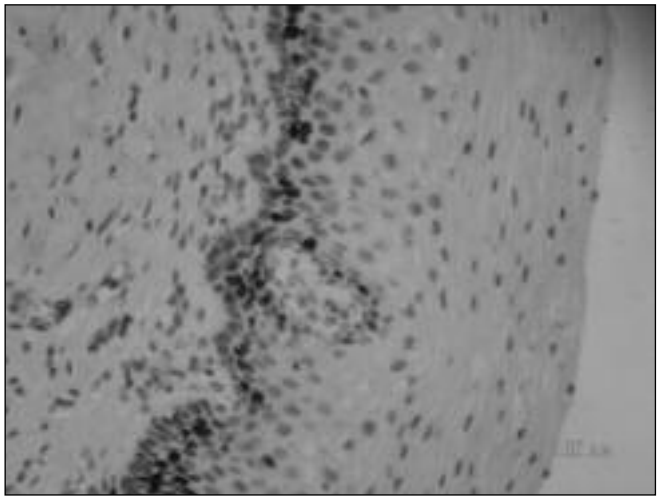

Figure 1. Ki-67 positive cells only in the parabasal area in normal cervix (IHK, x50).

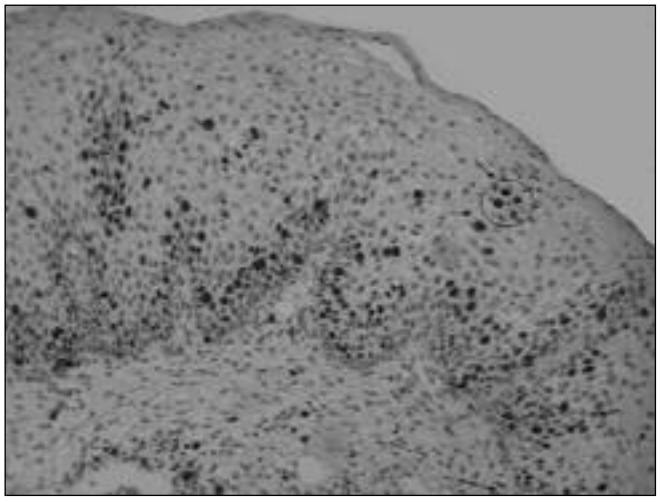

Figure 2. Ki-67 positive cells in the lower third of the epithelial thickness and positive cell cluster (arrow shows the cicled positive cell clusters) in the upper two thirds of the epithelial thickness in low-grade SIL (IHK, x50).

Ki-67 positive cells (Ki-67 index) were between 100/400 -264/400 in low-grade SIL cases (Table 1). Mean value was 153.5±50. In normal cases, Ki-67 positive cells ranged between 36/400-100/400. Mean value was 72.1±24.8. Mean value of Ki-67 positive cells in low-grade SIL cases was significantly higher than those of normal cases (p<0.05) (CI was 95%) (Table 2).

While Ki-67 positive cell clusters were observed in 10 (77%) of the 13 low-grade SIL cases (Table 1), none of the normal cases was positive for Ki-67 cell clusters (p<0.05) (Table 2). Specificity was 100% and sensitivity was 70% for Ki-67 positive cell clusters.

In the present study, two different antigen retrieval solutions, citrate buffer and EDTA, were applied to all samples to improve staining results of Ki 67. In our laboratory conditions antigen retrieval with citrate buffer has not been successful for Ki-67, therefore, antigen retrieval with EDTA has also been used. While only negative to weak staining was obtained with citrate buffer, sections treated with EDTA demonstrated strong positive reactivity.

Table 1. The diagnosis after reevaluation, Ki-67 index and Ki-67 cell clusters in low-grade SIL cases

Case no	Diagnosis after reevaluation	Ki-67 Index	Ki-67 Cell Clusters
3	Low-grade SIL	145/400	Positive
6	Low-grade SIL	200/400	Positive
7	Low-grade SIL	140/400	Positive
10	Low-grade SIL	264/400	Positive
12	Low-grade SIL	130/400	Positive
15	Low-grade SIL	113/400	Positive
24	Low-grade SIL	123/400	Negative
25	Low-grade SIL	120/400	Positive
26	Low-grade SIL	235/400	Positive
27	Low-grade SIL	170/400	Negative
29	Low-grade SIL	130/400	Positive
33	Low-grade SIL	100/400	Negative
34	Low-grade SIL	125/400	Positive

Discussion

SIL comprises biologically heterogeneous group of lesions of cervix. It is well known that increasing SIL correlates with increasing higher grades and/or invasive carcinoma. In many surgical pathology practices, it is important to determine

Table 2. The diagnosis after reevaluation, Ki-67 index and Ki-67 cell clusters in normal cases

Case no	Diagnosis after reevaluation	Ki-67 Index	Ki-67 Cell Clusters
1	Normal	100/400	Negative
2	Normal	96/400	Negative
4	Normal	40/400	Negative
5	Normal	42/400	Negative
8	Normal	36/400	Negative
9	Normal	95/400	Negative
11	Normal	85/100	Negative
13	Normal	40/400	Negative
14	Normal	50/400	Negative
16	Normal	40/400	Negative
17	Normal	70/400	Negative
18	Normal	100/400	Negative
19	Normal	55/400	Negative
20	Normal	98/400	Negative
21	Normal	100/400	Negative
22	Normal	100/400	Negative
23	Normal	100/400	Negative
28	Normal	81/100	Negative
30	Normal	65/100	Negative
31	Normal	61/400	Negative
32	Normal	60/400	Negative

both SIL and its grade (2,3). In addition, there has been problem in distinguishing SIL from nonneoplastic lesions such as immature metaplasia, atrophy and secondary changes to cervicitis as well as the normal cervix. Thus, over and under treatment as well as unnecessary controls can be the result. Moreover, patient may be subjected to unnecessary discomfort, inconvenience and emotional trauma. To prevent misdiagnosis, pathologists should be cautious in detecting SIL lesions (2-5,7).

Histological assessment remains the basis for the treatment and the follow-up. Inconsistent application of morphologic criteria compounded by the changes mimicking SIL, however, may lead to misdiagnosis (2-5,7). The subjectivity of diagnosis mainly rests upon identification of the cytopathological effects of human papillomavirus (HPV). To confirm the diagnosis, HPV DNA detection is necessary. However, because of the high cost, it is not routinely performed in tissue sections. Particularly in formalin fixed paraffin embedded tissues, it is not suitable for daily practice (1,2,5,6,8,11). In our study, we have observed koilocytic changes in the tissue sections however it has not been possible to demonstrate the presence of HPV because of our laboratory conditions. The cases were diagnosed as LSIL when both the cellular changes in the epithelium and the koilocytosis, which indicates the cytopathological effect of HPV, were demonstrated.

Additional methods are therefore required to avoid misdiagnosis of normal cervix, non-neoplastic epithelial lesions and SIL (1,2,4,5,8). Recent studies have shown that immunostaining with Ki-67, a cell proliferation marker, can be useful in identifying HPV related lesions of the cervix. It is useful when the morphological features are not diagnostic for the lesion and also, it is accepted as an independent reference for improving the diagnosis of SIL. Ki-67, being an easy, reproducible and inexpensive test, is widely used in pathology laboratories especially in paraffin embedded tissues. In normal cervix, Ki-67 positive cells are confined to the parabasal cell layer. Moreover, the percentages are low and range between 7% and 14%. In SIL, its expression increases in the parabasal areas with diffuse extension of the positive cells into the intermediate and superficial epithelial layers with an increasing grade of the cytological changes (6,8,10,11,13,14).

In this study Ki-67 index of normal cervix was lower than that of low-grade SIL. While normal percentages of the normal cervix ranged between 9% and 24%, in low-grade SIL the index was 25-66%. We found high Ki-67 index in low-grade SIL, which was in agreement with the reported studies (4,7,8,10). Inadequate sample size is the limitation of our study.

Recently, Pirog et al. showed that the presence of a cluster of at least two nearby Ki-67 positive nuclei in the upper two thirds of the epithelial thickness could be useful as a diagnostic criterion for non-SIL and low-grade SIL cases (5). Moreover, the Ki-67 positive cell cluster is used as a quality control. In many previous studies Ki-67 staining was determined quantitatively, by calculating the percentage of Ki-67 positive nuclei to all nuclei, by either manual counting or by computer image analysis. This technique, however, is seen as not only time consuming but also cumbersome. Sometimes counting Ki-67 does not allow clear discrimination of normal cases from low-grade SIL. Other studies have shown that Ki-67 positive cell cluster presence is highly sensitive and specific for this discrimination (5,10).

In our study, sensitivity and specificity of Ki-67 positive cell cluster was found 70% and 100%, respectively. The Ki-67 positive cell cluster, as well as the Ki-67 index, helped us make accurate diagnoses in four cases. In this study, although both Ki-67 index and positive cell cluster improved our results, we concluded that Ki-67 positive cell cluster seems more reliable and easier. Application of quantitative techniques is subject to individual interpretation. In addition, interpretation may be influenced by epithelial orientation relative to section plane, in which case tangential sectioning may result in over-diagnosis (2,4). Moreover, while some authors count both the strongly and the weakly staining Ki-67 positive cells to calculate the Ki-67 index, others count only the strongly positive ones. Thus, there have been differences in the Ki-67 index for the same lesions. However, the Ki-67 positive cell cluster is a more prominent feature, not subject to individual interpretation. Furthermore, Ki-67 staining results could change according to laboratory conditions. Despite the recommended citrate buffer solution to improve staining of the cell nuclei with Ki-67, EDTA as an antigen retrieval was more reliable to improve Ki-67 staining. Therefore, negatively stained cases should also be treated with EDTA before making accurate diagnosis. Especially when the cases, inconsistently with the morphologic findings, fail to stain with Ki-67, pathologists should be careful in making the final diagnosis. Because having a diagnosis of low-grade SIL can be a serious burden for a patient.

In conclusion, Ki-67 might be an important and valuable additional technique for histopathological assessment of the cervix and the Ki-67 positive cell cluster evaluation might be easy and reproducible. In evaluating the positivity of Ki-67 staining, to prevent over- or under-diagnosis, laboratory conditions should also be considered.

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