Re-evaluation of Negative Cone Biopsy Results with Ki-67 and p16 Immunostaining following Positive Cervical Biopsy

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Abstract

Background: Cervical conization is a standard diagnostic method for precancerous lesions. However, its results could be negative despite an initially positive punch biopsy. The present study aimed to re-evaluate pathological biopsies with Ki-67 and p16 immunostaining to assess the diagnostic accuracy of punch biopsies.

Methods: This retrospective study performed in Motahhari Clinic and Shahid Faghihi Hospital, (Shiraz, Iran). 88 punch and cone biopsy slides from 2007-2016 were re-evaluated by two pathologists, and the results were compared with the original diagnoses. Agreement between the initial diagnoses and re-evaluations and between our pathologists were assessed with the kappa coefficient. Twenty-two negative conization results after positive punch biopsy were re-sectioned and evaluated with Ki-67 and p16 immunostaining.

Results: The overall agreement (kappa) between the primary punch diagnoses by the original pathologists and those made in the present study (by the first and second pathologists) before immunohistochemical (IHC) staining was 0.33 and 0.43, respectively. The kappa coefficient between punch biopsy diagnoses by the first and second pathologists before IHC staining was 0.73, while it increased to one after IHC staining with Ki-67 and p16. Out of the 22 specimens with the positive punch and negative cone biopsies on initial diagnosis, cervical intraepithelial neoplasia (CIN) was not confirmed in 11 specimens by our pathologists after IHC staining with Ki-67 and p16. These cases were reclassified as transitional metaplasia or acute/chronic cervicitis.

Conclusion: Punch biopsy can be misdiagnosed as CIN positive, leading to unnecessary conization. The use of Ki-67 and p16 markers as appropriate ancillary tests are recommended.

Keywords: Cervix uteri • Conization • Cervical intraepithelial neoplasia • Immunohistochemistry

Introduction

As the fourth most prevalent type of cancer among women and the third deadly cancer worldwide among all other types of cancer, cervical cancer is a major global health concern, putting a huge financial burden on the affected patients. More
than 85% of cervical cancer cases are reported in developing countries. In Iran, its prevalence has been on the rise during the last decades.\(^1\) It is believed that early detection of cervical intraepithelial neoplasia (CIN) is critical in reducing the likelihood of developing cervical cancer. CIN is the precancerous epithelial transformation of invasive squamous cell carcinoma of the cervix. The role of human papillomavirus (HPV), a DNA virus, in causing cervical cancer and precancerous lesions is well recognized especially the high-risk types. The E6 and E7 viral oncoproteins can bind to host cell regulatory proteins and inactivate tumor suppressor P53 and Rb genes, respectively, leading to cell proliferation and increase of cell mutation. An immunohistochemical (IHC) study can reveal cellular dysregulation of antibodies such as Ki-67 and p16.\(^2\)-\(^4\)

Cell proliferation in the G1-S phase is regulated by p16, which negatively impacts cell proliferation. There is a reciprocal relationship between p16 and pRb, which is a tumor suppressor protein. Inactivity of pRb results in the overexpression of p16 which, commonly presents in HPV infection.\(^3\) Ki-67 is a nuclear protein expressed in the active phases of the cell cycle (G1, S, G2, and M phases), and its overexpression causes high cellular proliferation, commonly observed in HPV infection.\(^3\) Therefore, examining the expression of biomarkers such as p16 and Ki-67 in pathological biopsies has been suggested as a method with high sensitivity to improve diagnostic accuracy.\(^4\)

Cervical conization is a widely used and efficient intervention in the diagnosis and control of precancerous cervical lesions. Its main advantages are low blood loss, shorter operating time, low cost, and high success rate.\(^5\) The results of cervical conization could be negative despite the diagnosis of precancerous lesions on punch biopsy. The present study aimed to evaluate diagnostic accuracy and agreement between pathologists by adjunctive Ki-67 and p16 IHC staining.

### Materials and Methods

#### Study Design

In the current retrospective study, 118 pathology slides of formalin-fixed, paraffin-embedded specimens of cervical punch and cone biopsies were re-evaluated. The specimens were obtained from the pathology archives (dated 2007-2016) of Motahari Clinic and Shahid Faghihi Hospital, both affiliated to Shiraz University of Medical Sciences, Shiraz, Iran. The protocol of the study was approved by the Ethics Committee of Shiraz University of Medical Sciences (number: IR.SUMS.MED.REC.1395.S181). All punch biopsies had been performed under colposcopic guidance, and all conization samples were taken via the loop electrosurgical excision procedure (LEEP) method.

Out of the 118 specimens, 30 were excluded due to the missing data, unavailable slides, or tissue blocks. The remaining 88 punch and cone biopsy slides were evaluated. These slides were re-examined by two pathologists, unaware of the primary diagnosis, and the discrepancy between the initial diagnosis and re-evaluation was confirmed. Agreement between the initial diagnoses and re-evaluations as well as between the two pathologists was assessed with the kappa coefficient. The initial diagnosis with hematoxylin and eosin (H&E) staining indicated 22 patients with negative cone biopsy results following a positive punch biopsy. Cone biopsies of the 22 specimens initially diagnosed as negative for dysplasia were completely sectioned, reviewed, and suspicious areas were stained with Ki-67 and p16. The absence of intraepithelial squamous, glandular neoplasia, or invasive disease in the cone biopsy specimens was defined as negative. The patients’ age, the interval between punch and cone biopsy, the presence of transformation zone (TZ) in the cone biopsy samples, the method of conization, and denudation of squamous epithelium were recorded. Moreover, the corresponding punch biopsies were stained with Ki-67 and p16. The agreement between the two pathologists on the IHC study was assessed with the kappa coefficient.

#### Immunohistochemical Staining

IHC staining for Ki-67 and p16 antigens was performed on 5-µm sections of formalin-fixed, paraffin-embedded blocks using the avidin-biotin-peroxidase complex method. Unstained tissue sections were coated on Poly-L-lysine slides (Samaatashkhis, Iran) for IHC staining, deparaffinized with xylene (Merck, Germany) for 30 minutes, and gradually rehydrated with alcoholic ethanol (100%→96%→70%, every 20 seconds). They were placed in distilled water for two minutes, followed by a mixture of distilled water and H\(_2\)O\(_2\), and washed in phosphate-buffered saline (PBS; Samaatashkhis, Iran), for five minutes. All slides were boiled in Tris buffer (PH=9.0; Samatashkhis, Iran) for 50 minutes, then cooled and put in PBS for five minutes. The slides were blocked with 10% goat serum and incubated for 20 minutes at room temperature, and then incubated in a humidified chamber for one hour. The following antibodies were used:
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Monoclonal rabbit anti-Ki-67 antigen, clone SP6 (Dako, code: N1633, USA; ready to use), and mouse monoclonal anti p16INK4a (Biogenex, clone G175-405, USA; diluted in PBS).

The slides were washed in PBS for 20 minutes and incubated in a wet chamber. Then, one drop of HRP polymer was applied to the sections for 30 minutes at room temperature and washed in PBS buffer for 10 minutes. 3,3’ Diaminobenzidine (DAB) chromogen (Dako, code K3468, USA) was added, and then the slides were washed in PBS for five minutes. The slides were counterstained with hematoxylin (Dako, code: CS70030-2, USA; ready-to-use), rinsed under running water for some minutes, dehydrated in graded ethanol solutions, cleared with xylene, and mounted.

Immunohistochemical Scoring

To identify the precise location of the lesions, the IHC stained sections were examined alongside the H&E stained slides. Ki-67 (MIB1) staining was classified as positive when a cluster of at least two strongly stained epithelial nuclei was present in the upper two-thirds of the epithelial thickness anywhere within the lesion. Parabasal cells staining was used as an internal positive control.

The p16 was classified as positive when nuclear and continuous diffuse cytoplasmic staining of the cells appeared in the basal and parabasal cell layers of the squamous epithelium and reached an intermediate and superficial cell layer mostly recognized by diffuse staining pattern. It was considered negative when completely unstained or revealing focal or sporadic epithelial staining, especially not of the basal and parabasal cells. The distribution of immunoreactive cells was used as a basis to evaluate the scoring of IHC results. CIN III slides were employed as positive controls. To avoid subjective interpretation, it was decided that staining intensity not be graded.

Statistical Analysis

Data were analyzed using SPSS software (version 21.0). Quantitative and categorical variables were presented as mean±SD and frequency (percentage), respectively. The inter-observer reliability was assessed using the kappa coefficient. P<0.05 were considered as statistically significant.

Results

The mean age of the patients was 39±10.2 years (25-78 years). The mean interval between punch and cone biopsy was 8.5 weeks. The results of the initial diagnosis of cone and punch biopsies on H&E slides are shown in table 1. The overall agreement between the punch diagnoses by the original pathologists and those in the present study (the first and second pathologists) before IHC was 0.33 (SE: 0.051, P=0.001) and 0.43 (SE: 0.058, P=0.001), respectively. The kappa coefficient between punch biopsy diagnoses by the first and second pathologists before IHC staining was 0.73 (SE: 0.061, P=0.001).

The overall agreement between the first cone diagnosis by the original pathologists and our pathologists prior to IHC staining with Ki-67 and p16 was 0.47 (SE: 0.057, P=0.001) and 0.48 (SE: 0.061, P=0.001), respectively. The kappa coefficient between cone biopsy diagnoses by our first and second pathologists before IHC staining with Ki-67 and p16 was 0.79 (SE: 0.056, P=0.001). The discrepancy between initial diagnosis by the original pathologists and consensus diagnosis is shown in table 2. Agreement between the diagnoses of CIN I, II, and III by the first and second pathologists before IHC staining was 0.75 (CIN I vs. CIN II), 0.61 (CIN I vs. CIN III), 0.65 (CIN II vs. CIN III), and after IHC staining with Ki-67 and p16 was 1 (SE: 0.062, P=0.001).

Out of the 22 specimens with a positive punch biopsy and subsequent negative cone biopsy, 11 specimens (group 1) had CIN diagnosis in the initial punch diagnosis, although CIN was not confirmed by our pathologists. After IHC staining with Ki-67 and p16, out of the 10 specimens, which were at first diagnosed with high-grade CIN, one was reclassified as transitional metaplasia, and the remaining specimens were...

| Primary punch diagnosis | Negative N (%) | CIN I N (%) | CIN II N (%) | CIN III N (%) | Total N (%) |
|-------------------------|---------------|------------|-------------|--------------|-------------|
| Negative                | 13 (72.2%)    | 2 (11.1%)  | 2 (11.1%)   | 1 (5.6%)     | 118 (100.0%)|
| CIN I                   | 3 (27.3%)     | 8 (72.7%)  | 0 (0.0%)    | 0 (0.0%)     | 111 (100.0%)|
| CIN II                  | 14 (58.4%)    | 5 (20.8%)  | 3 (12.5%)   | 2 (8.3%)     | 224 (100.0%)|
| CIN III                 | 5 (14.2%)     | 3 (8.6%)   | 1 (2.9%)    | 26 (74.3%)   | 335 (100.0%)|
| Total                   | 35 (39.8%)    | 18 (20.4%) | 6 (6.8%)    | 29 (33.0%)   | 88 (100.0%) |

CIN: Cervical intraepithelial neoplasia
reclassified as acute and/or chronic cervicitis (figures 1 and 2). The one specimen, initially diagnosed as low-grade CIN, was reclassified as acute and/or chronic cervicitis (table 3). The second group consisted of 11 specimens with CIN diagnosis was also confirmed by our pathologists. However, after IHC staining with Ki-67 and p16, three specimens with high-grade CIN were reclassified as low-grade CIN. The diagnosis of six specimens with high-grade CIN and two with low-grade CIN were confirmed in the re-evaluation (table 3).

Conization slides of the 22 specimens were reviewed. Of these specimens, five showed low-grade CIN, and one was classified as high-grade CIN. The remaining specimens showed no precancerous lesions in all sections and were classified as acute and/or chronic cervicitis.

Table 2: The discrepancy between primary and consensus diagnosis of cervical punch biopsy

| Primary cervical biopsy diagnosis | Final cervical biopsy diagnosis | Total N (%) |
|----------------------------------|---------------------------------|-------------|
|                                  | CIN I N (%)                     | CIN II N (%) | CIN III N (%) |
| Negative                         | 6 (33.3%)                       | 12 (66.7%)  | 0 (0.0%)       | 18 (100%)    |
| CIN I                            | 2 (18.2%)                       | 8 (72.7%)   | 0 (0.0%)       | 11 (100%)    |
| CIN II                           | 13 (54.2%)                      | 3 (12.5%)   | 2 (8.3%)       | 24 (100%)    |
| CIN III                          | 2 (5.7%)                        | 2 (5.7%)    | 0 (0.0%)       | 35 (100%)    |
| Total                            | 23 (26.1%)                      | 25 (28.4%)  | 2 (2.4%)       | 88 (100%)    |

CIN: Cervical intraepithelial neoplasia
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There was no significant difference between the mean age of the first and second groups (36 years vs. 35 years), respectively (P=0.375). The mean interval between the punch and cone biopsy procedures in the first and second groups was seven and ten weeks, respectively (P=0.385). The transformation zone was absent in two specimens (9%), and epithelial denudation was seen in six specimens (27.2%; first group: n=4, second group: n=2). There was no recurrence in 18 patients who were followed up between 1 and 8 years after conization (table 3).

Discussion

The results showed that 40% (n=35) of the initial diagnosis of the assessed conization specimens had a negative cone biopsy result. Of those with a positive punch biopsy, 25% (n=22) had a negative cone biopsy result. Other studies have reported different percentages for negative cone biopsy following a positive punch biopsy, varying from 15.5% to 25%.6-8 The difference in the results could be due to the absence of lesion in the conization specimen and the number of quadrants involved.9 Despite the differences, all studies point to negative LEEP results as a common phenomenon. Since its recurrence rate is similar to positive cases, it has been recommended to monitor cases that have been tested negative in the same way as the positive cases.5

In the current study, re-evaluation of the negative cone biopsies with Ki-67 and p16 IHC
staining showed positivity in 68.2% and 27.7% of the cases, respectively. Of the 22 specimens, five were classified as low-grade CIN and one was high-grade CIN. In line with previous studies, our results also indicated that Ki-67 and p16 are valid, simple, available, reproducible, and low-cost biomarkers to improve diagnostic accuracy.\(^\text{1-12}\)

Our study revealed that 11 out of 22 initially positive punch biopsies were re-evaluated negative; therefore, unlike cone biopsy, further tests were not required. A surgical procedure is not indicated for low-grade CIN. After IHC staining with Ki-67 and p16, three specimens with primary high-grade CIN were reclassified as low-grade CIN. This was in line with previous studies indicating the low sensitivity of punch biopsy in the case of high-grade dysplasia.\(^\text{13}\). Nonetheless, re-evaluation of biopsies by different pathologists, contingent upon high inter-observer agreement, could improve the diagnostic accuracy of the punch biopsy.

Aslani and colleagues reported that the re-evaluation of cervical biopsies by two pathologists originally diagnosed as 31 negative CIN and 46 positive CIN changed to 54 negative CIN and 23 positive CIN. The reported sensitivity and specificity for Ki-67 were 95.6% and 85.1%, respectively, and for p16, they were 91.3% and 98.1%, respectively.\(^\text{2}\) Palma and colleagues also evaluated cervical colposcopy-guided biopsy slides diagnosed as CIN by two pathologists and reported that 7.4% of women with CIN I or less severe were re-evaluated as CIN II or higher grades. However, approximately 15% of the cases initially diagnosed as CIN II or higher were subsequently downgraded to CIN I or less severe.\(^\text{15}\) These results confirmed our findings on the effectiveness of Ki-67 and p16 biomarkers on cervical biopsy slides to improve diagnostic accuracy. Therefore, it is recommended to test these biomarkers on cervical punch and conization specimens.

In the present study, limited transformation zone was seen in 9% (n=2) and epithelial denudation in 27.2% (n=6) of the specimens. In a study by Livasy and colleagues, the absence of the transformation zone or denudation of negative LEEP was seen in only 2% and 3% of the cases, respectively. The most common limiting histological feature in negative LEEP was an extensive cautery artifact that impaired the evaluation of small foci of dysplasia.\(^\text{16}\) Therefore, preventing some factors such as the lack of the transformation zone sampling, epithelial denudation, and hemorrhage could be important steps in reducing the specimen’s artifact. A number of theories have been suggested to help explain why lesion is absent in the colonization specimen, while a previous biopsy confirmed CIN. Either the initial biopsy might have entirely removed the small cervical lesions\(^\text{16-18}\) or spontaneous regression of the residual lesion following the punch biopsy might have been the cause; estimated 60-63% for low-grade CIN (CIN I), 40-74% for CIN II, and 24-33% for CIN III.\(^\text{19}\) The factors related to an increased rate of regression include young age and long duration between biopsy and conization.\(^\text{17}\) However, we found no differences in age between the patients with and without lesion in conization specimens. Thirdly, the lesion area may have been missed in histological sections. The other causes include failure to remove dysplasia during the LEEP or not including the lesion in the surgical specimen due to the high location of squamocolumnar junction in the endocervical canal. The main cause of the discrepancies between the diagnoses of cervical biopsy and cervical conization in our study was the misdiagnosis of a cervical punch biopsy. The most common differential diagnoses of CIN III include acute/chronic inflammation, reactive or regenerative changes, basal cell hyperplasia, mature or immature squamous cell metaplasia, and atrophic epithelium.\(^\text{20}\)

Among the 88 eligible slides evaluated initially, 18 (20.5%) had negative punch biopsy results. This finding is in line with the high false-negative and false-positive results reported previously for a punch biopsy.\(^\text{14, 17, 21}\) Sorby and colleagues also reported 23.8% of negative cases diagnosed as CIN II in follow-up biopsies.\(^\text{22}\) This is in line with our findings on the low sensitivity of punch biopsy since 50% (n=8) of the negative cases were reclassified as low-grade CIN after re-evaluation. The results of the re-evaluation of the punch biopsies by two pathologists and the IHC study indicated that the diagnosis of 17% (n=15) of the specimens with high-grade CIN and two with low-grade CIN changed to negative for dysplasia. Whereas, 5.6% (n=5) with high-grade CIN changed to low-grade CIN, and one specimen with low-grade CIN changed to high-grade CIN. Therefore, our results confirmed previous studies reporting punch biopsy as an unreliable tool for decision-making, suggesting further examinations for definite diagnosis.\(^\text{15, 14}\)

Another important aspect of the present study was that despite a high agreement between our pathologists (>0.7), the agreement between the pathologists’ reports and the initial diagnosis was low (<0.5) for both punch and cone biopsies. Previous studies have also shown a high inter-observer variability for CIN assessment.\(^\text{2, 23}\) Basu and colleagues reported poor overall agreement (k=0.36) with the lowest agreement in the low-grade squamous intraepithelial lesion.
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category (κ=0.23) as well as the highest one in the squamous cell carcinoma (κ=0.76). This is in line with our results indicating a high inter-observer variability and low agreement for H&E stained slides. Therefore, we recommend IHC as an effective detection method with high reproducibility. In terms of punch biopsy samples, we recommended obtaining detailed information about the reasons for sampling and the results of Pap smears. In case of a discrepancy between the punch biopsy diagnosis and Pap smear report, a review of Pap smear slides is recommended. The main limitations of the present study were the low sample size and unavailability of Pap smears of cases with both negative punch and cone biopsy results.

Conclusion

Both punch and LEEP conization biopsies could be associated with high inter-observer variability between pathologists. Negative LEEP result following a positive initial cervical biopsy could be due to the misdiagnosis of the primary punch biopsy. We found that acute/chronic inflammation, reactive or regenerative changes, basal cell hyperplasia, mature or immature squamous cell metaplasia, and atrophic epithelium mimicked CIN. The use of ancillary diagnostic methods such as IHC study, an adjunct to H&E staining could resolve some ambiguous cases. Since most patients were of reproductive age, in order to prevent unnecessary invasive treatment, the use of Ki-67 and p16 markers as complementary tests, is recommended to differentiate dysplastic and non-dysplastic lesions, especially in the suspicious cases with high-grade CIN.

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