OBJECTIVE: To evaluate the usefulness of p16INK4a (p16) and Ki-67 staining in high-grade cervical intraepithelial neoplasia (CIN2) biopsies in order to predict CIN3 results in cone specimens, thereby sparing those not likely at risk for CIN3 from unnecessary cone excision.

STUDY DESIGN: We retrospectively recruited patients with CIN2 colposcopy-directed biopsy treated by loop electrosurgical excision procedure. The expression of p16 and Ki-67 was qualitatively and quantitatively analyzed in all biopsies and cone specimens.

RESULTS: A total of 123 patients from January 2009 to December 2010 were included in the study. CIN3 in cone specimens was observed in 35 patients (28.5%). Ki-67 positive immunostaining in >50% of epithelial cells was related to CIN3 diagnoses in cone specimens (p=0.043). However, p16+ and Ki-67+ evaluated by thirds of the epithelial thickness in CIN2 biopsies did not show a significant correlation with the cone results. In multivariate analysis, Ki-67 cell expression over 50% in CIN2 biopsies and high-grade squamous intraepi-
The lesion (HSIL) in the previous cytology were statistically associated with CIN3 results in the cone (odds ratio [OR] 2.55, 95% confidence interval [CI] 1.04–6.29; OR 2.68, 95% CI 1.07–6.72, respectively).

CONCLUSION: Patients with HSIL in the previous cytology and Ki-67 cell expression over 50% in their CIN2 biopsies could be considered in need of treatment by cone for their higher risk of underlying CIN3 lesions.

Keywords: cervical intraepithelial neoplasia 2, CIN2, cervical intraepithelial neoplasia 3, CIN3, high-grade squamous intraepithelial lesion, HSIL, immunohistochemistry, Ki-67, p16, p16INK4a.

Cervical intraepithelial neoplasia grade 2 (CIN2) is an intermediate state of cervical pathology between CIN1 and CIN3. Currently, some authors propose to consider CIN2 and CIN3 all together as high-grade squamous intraepithelial lesion (HSIL), particularly when p16INK4a (p16) staining is positive for their interpretation as a precancerous lesion. However, not all HSIL and p16-positive lesions will progress. Around 40–60% of CIN2 lesions are likely to regress to CIN1 or less, while 10–20% progress to CIN3 and 30–40% persist to CIN2 in young patients after 1–3 years. Historically, most patients with CIN2 diagnoses have undergone cervical excisional treatment, which increases obstetric complications. Considering that some of those patients with CIN2 could have regressed spontaneously, the latest consensus of the American Society for Colposcopy and Cervical Pathology (ASCCP) recommend expectant management and follow-ups for young women with CIN2-confirmed biopsy.

Complementary biomarkers in both cytology and histology such as p16 and Ki-67 could improve the positive predictive value of cervical screening results. p16 is a tumor-suppressor protein; it is a biomarker of human papillomavirus (HPV) oncogenic activity because it is a cell-cycle regulator that inhibits the activity of cyclin-dependent kinases, which phosphorylate the retinoblastoma protein. The monoclonal antibody Ki-67 detects a nuclear protein, the expression of which indicates epithelial cell proliferation, and it increases in HPV infection. Most studies on the topic of p16 and Ki-67 expression report a significant positive correlation with the severity of CIN, and it is used to improve interobserver accuracy between pathologists.

Some follow-up studies have reported the use of p16 and Ki-67 expression in cervical biopsies—mainly CIN1 lesions—to identify patients with an increased risk for progression to high-grade lesions. However, evidence is still scarce in terms of evaluating the precise role of p16 and Ki-67 analysis in CIN2 biopsies.

On the basis of this knowledge, we designed a study to evaluate the role of p16 and Ki-67 staining in CIN2 biopsies to predict the presence of CIN3 in cone results.

Materials and Methods

Study Population

In this retrospective cohort study we included patients diagnosed with CIN2 using a colposcopy-directed biopsy and followed by cone excision over the period from January 2009 to December 2010 at the Department of Obstetrics and Gynecology, Parc de Salut Mar–Hospital del Mar, Barcelona.

Inclusion criteria were women >18 years old with colposcopy-directed biopsy diagnosed with CIN2 available for immunohistochemical staining treated by loop electrosurgical excision procedure (LEEP) in our hospital. Exclusion criteria were HIV positivity, women previously treated by hysterectomy or other cervical procedures, and women treated in another hospital.

Certified obstetrician-gynecologists who practice colposcopy routinely obtained cervical samples. One or two colposcopy-directed biopsies were taken from the most severe and suspicious areas. Historically, most patients with CIN2 diagnoses have undergone cervical excisional treatment, which increases obstetric complications. Considering that some of those patients with CIN2 could have regressed spontaneously, the latest consensus of the American Society for Colposcopy and Cervical Pathology (ASCCP) recommend expectant management and follow-ups for young women with CIN2-confirmed biopsy.

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Histologic diagnoses were established using pure morphological criteria based on H&E staining. Two experienced gynecological pathologists (F.A. and B.L.) independently examined in a blinded fashion all specimens. Discordant results were discussed and a consensus for all diagnoses was obtained.

**Immunohistochemical Staining**

Immunohistochemical interpretation for p16 and Ki-67 of all biopsies and cone specimens was performed in a blinded manner. The p16 expression was analyzed using the CINtec Histology Kit (Ventana Medical Systems, Inc., Tucson, Arizona, U.S.A.), and Ki-67 was studied with prediluted antibody, clone 30-9 (Ventana Roche Diagnostics, Tucson, Arizona, U.S.A.). The samples were processed using an automatic process with Benchmark XT (Ventana Roche Diagnostics). Diffuse, continuous, and strong block-positive nuclear and cytoplasmic p16 staining across the epithelium involving at least one-third of the epithelial thickness (basal and parabasal layers) was considered a positive result. Focal, weak, and irregular expression was considered negative or non-block-positive staining (Figure 1A). For the positive cases, p16 was quantitatively analyzed in 3 epithelial layers: lower third of the epithelium (Figure 1B), two-thirds (Figure 1C), and more than two-thirds to up to full epithelial thickness (Figure 1D). Ki-67 expression was evaluated by nuclear staining of epithelial cells. It was considered negative when positive cells were restricted to only the suprabasal cell layer or when no immunopositivity expression was detected (Figure 1E). Ki-67 was considered positive when immunopositivity was observed across the lower third of the epithelial layer (Figure 1F), the lower two-thirds (Figure 1G), or more than the lower two-thirds to up to full epithelial thickness (Figure 1H). Furthermore, we evaluated the percentage of epithelial cells showing Ki-67 nuclear staining independent of their placing across the epithelium.

**HPV Detection in Biopsy**

HPV detection and genotyping was performed only in biopsy material. DNA was extracted from two 15-micron sections of paraffin-embedded tissue using the QIAmp Tissue Kit (Qiagen GMBH, Hilden, Germany) following the manufacturer’s protocol. HPV detection and typing was performed using the Linear-Array (LA) Genotyping Test (Roche Diagnostics, Mannheim, Germany). This method involves DNA-PCR followed by hybridization using a reverse line blot system to simultaneously detect 37 HPV types (HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 65, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, 82 variant 39 or IS39- and 89 -CP6108-). The test is based on amplification of the 450 bp in the L1 region using PGMY primers, and it also amplifies a region of the beta-globin gene as an internal control.

**Figure 1** Representative figures of immunohistochemical grading for p16 INK4a (A–D) and Ki-67 (E–H) in cervical intraepithelial neoplasia tissues. p16 INK4a nuclear and cytoplasmic staining: (A) negative, (B) lower one-third of the epithelium, (C) lower two-thirds of the epithelium, (D) more than two-thirds up to full epithelial thickness. Ki-67 nuclear staining: (E) negative, (F) lower one-third of the epithelium, (G) lower two-thirds of the epithelium, (H) more than two-thirds up to full epithelial thickness.
Statistical Analysis

The association of cone results with sociodemographic features, cytology, HPV-type infection, and p16 and Ki-67 expression in patients with CIN2 were evaluated using the Mann-Whitney U test for continuous variables and the $\chi^2$ test or Fisher’s exact test for categorical variables, when appropriate.

The risk of underlying CIN3 in cone results of different variables was assessed calculating the odds ratio (OR) and 95% confidence intervals (CIs). Variables that were found statistically significant in the univariate analysis were considered for multivariate logistic regression analysis to determine factors that improve the prognostic value of individual features.

All statistical tests were performed with two-sided tests and considered significant at $p$ value <0.05. SPSS 18.0 (SPSS, Inc., Chicago, Illinois) was used in all analyses.

Results

A total of 123 patients with CIN2 biopsy treated by cone were included. Their cone results were as follows: 29 with CIN1, 57 with CIN2, 35 with CIN3, and normal for 2. Invasive cervical cancer was not found in any specimen. The mean (standard deviation) interval time between CIN2 biopsy and cone excision was 3.18 (1.60) months and did not exhibit a statistically significant association with the cone results according to biopsy-cone interval ($p=0.369$).

Sociodemographic features of the study population and their association with the cone results are presented in Table I. There were no significant differences between the groups.

Cytological and histological analysis according to cone results classified as CIN3 versus CIN2 or less ($\leq$CIN2) is presented in Table II. The prevalence of p16 positive in CIN2 biopsies was 92.7%, with no significant differences to cone results (94.3% CIN3 vs. 92.0% $\leq$CIN2, $p=0.667$). Only 9 (7.3%) of the CIN2 biopsies were p16 negative, 7 resulted in $\leq$CIN2 in cone biopsy, and 2 were diagnosed with CIN3. When we graded by thirds the epithelial distribution of p16 expression in CIN2 biopsy, we did not find association with the cone results.

In reference to Ki-67 immunostaining, we reported that 98.4% of CIN2 biopsies were Ki-67 positive. The 2 Ki-67–negative cases were CIN1 in the cone results. When grading the epithelial distribution of Ki-67 by thirds in CIN2 biopsies, we did not find a significant association to cone results. However, a Ki-67 cell expression over 50% in CIN2 biopsies was statistically significantly associated to CIN3 in cone specimens as compared to

Table 1 Characteristics of the Study Population According to Cone Results

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total population (n = 123)</th>
<th>$\leq$CIN2 (n = 88)</th>
<th>$\leq$CIN3* (n = 35)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs, mean (SD)</td>
<td>34.32 (7.89)</td>
<td>34.16 (7.74)</td>
<td>34.71 (8.34)</td>
<td>0.871</td>
</tr>
<tr>
<td>No. of lifetime sexual partners, mean (SD)</td>
<td>9.11 (5.59)</td>
<td>9.56 (5.59)</td>
<td>7.93 (5.60)</td>
<td>0.340</td>
</tr>
<tr>
<td>Age at first sexual intercourse yrs, mean (SD)</td>
<td>16.95 (2.24)</td>
<td>16.66 (2.19)</td>
<td>16.92 (2.47)</td>
<td>0.904</td>
</tr>
<tr>
<td>Active smoking, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>69 (62.7)</td>
<td>47 (61.8)</td>
<td>22 (64.7)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>41 (37.3)</td>
<td>29 (38.2)</td>
<td>12 (35.3)</td>
<td></td>
</tr>
<tr>
<td>Immunosuppression, n (%)b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4 (3.3)</td>
<td>2 (2.3)</td>
<td>2 (5.7)</td>
<td>0.332</td>
</tr>
<tr>
<td>No</td>
<td>119 (96.7)</td>
<td>86 (97.7)</td>
<td>33 (94.3)</td>
<td></td>
</tr>
<tr>
<td>Pregnancy, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4 (3.9)</td>
<td>2 (2.7)</td>
<td>2 (6.7)</td>
<td>0.349</td>
</tr>
<tr>
<td>No</td>
<td>99 (96.1)</td>
<td>71 (97.3)</td>
<td>28 (93.3)</td>
<td></td>
</tr>
<tr>
<td>Nulliparity, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>67 (59.8)</td>
<td>51 (65.4)</td>
<td>16 (47.1)</td>
<td>0.069</td>
</tr>
<tr>
<td>No</td>
<td>45 (40.2)</td>
<td>27 (34.6)</td>
<td>18 (52.9)</td>
<td></td>
</tr>
<tr>
<td>Contraceptive method, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hormonal</td>
<td>36 (35.3)</td>
<td>26 (36.6)</td>
<td>10 (32.3)</td>
<td>0.907</td>
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<tr>
<td>Condom</td>
<td>37 (36.3)</td>
<td>25 (35.2)</td>
<td>12 (38.7)</td>
<td></td>
</tr>
<tr>
<td>No method</td>
<td>29 (28.4)</td>
<td>20 (28.2)</td>
<td>9 (29.0)</td>
<td></td>
</tr>
</tbody>
</table>

SD = standard deviation.

*Adenocarcinoma in situ in 1 patient.

bNo HIV-positive patients.
Despite the fact that immunostaining epithelial distribution of p16 and Ki-67 by thirds in CIN2 biopsies were not associated with cone results, when we studied p16 and Ki-67 in the cone specimens we found that their distribution was significantly correlated with CIN grade (p<0.0001) (Figure 2). Indeed, p16 and Ki-67 expression in cone specimens with CIN1 were mostly observed within the lower third of the squamous epithelium, whereas in most cone specimens with CIN3 more than two-thirds were involved.

HPV genotyping was performed only in biopsies, not in cone specimens. The results are shown in Table II. Thirteen patients had insufficient material to perform HPV analysis. We found that patients

![Figure 2](image-url)
with HPV16 and/or HPV18 positive (HPV16/18) were significantly associated with CIN3 over ≤CIN2 in cone results (75.0% vs. 51.3%, p=0.022). Concerning previous cytological diagnoses, HSIL previous cytology was statistically significantly associated with CIN3 cone results as compared to ≤CIN2 (61.8% vs. 40.7%, p=0.037).

In the univariate analysis the risk for CIN3 as compared to ≤CIN2 in cone specimens was associated with over 50% Ki-67 cell expression (OR 2.30, 95% CI 1.02–5.19), HPV16/18 infection in CIN2 biopsy (OR 2.85, 95% CI 1.14–7.12), and HSIL previous cytology (OR 2.35, 95% CI 1.04–5.32) (Table III). In our advanced multivariate model, which was adjusted for all the significant variables in univariate analysis (namely over 50% Ki-67 cell expression and HSIL previous cytology), the association remained statistically significantly associated with the CIN3 cone result (OR 2.55, 95% CI 1.04–6.29; OR 2.68, 95% CI 1.07–6.72, respectively). In contrast, HPV16/18 positive was no longer significant (OR 2.50, 95% CI 0.96–6.56, p=0.062).

**Discussion**

Our study showed that a cell expression over 50% of Ki-67 in CIN2 biopsy was associated with CIN3 in cone results. However, p16 and Ki-67 expression, and grading in thirds the epithelial distribution of CIN2 biopsies, did not correlate with cone results. To our knowledge, this is the first study that evaluates p16 and Ki-67 in exclusively CIN2 biopsies to identify lesions that could have an underlying CIN3.

Previous follow-up studies have already shown that p16 might serve as a marker of risk of progression for CIN1 cases and HPV-positive women.17-19,22 Patients with p16-positive expression were shown to have an increased risk for CIN2+ (10–20%) as compared to patients with p16-negative (0–2%). The role of p16 in HSIL as a prognostic marker following treatment has been poorly evaluated. Although some authors correlated the expression of p16 in cone specimens as a marker of CIN grade,23 p16 did not appear to be useful as a prognostic value in predicting the clearance of high-risk HPV after the cone.24 Nevertheless, we analyzed the p16 expression in CIN2 biopsies and did not find association with subsequent cone results, possibly because p16 is only a marker of HSIL. For that reason we analyzed the p16 epithelial distribution expression by thirds in CIN2 biopsies to predict cone results, but we found no significant association. At present, as reference to the Lower Anogenital Squamous Terminology, p16

| Table III | Predictors of Underlying CIN3 in Cone Results: Univariate and Multivariate Analysis of Variables in Patients with CIN2 Biopsy |
|---|---|---|---|
| Variables | OR | 95% CI | p Value |
| **Univariate** | | | |
| Age (> 25 yrs) | 2.12 | 0.44–10.19 | 0.350 |
| No. of lifetime sex partners (> 5) | 0.45 | 0.13–1.54 | 0.202 |
| Age at first intercourse (> 18 yrs) | 1.80 | 0.36–9.08 | 0.476 |
| Active smoking | 1.13 | 0.49–2.63 | 0.774 |
| Immunosuppression | 2.61 | 0.35–19.25 | 0.346 |
| Multiparity (≥ 1 child) | 2.33 | 0.59–9.83 | 0.371 |
| Hormonal contraceptive use | 0.82 | 0.34–2.02 | 0.672 |
| Condom use | 1.16 | 0.49–2.78 | 0.735 |
| HSIL previous cytology | 2.35 | 1.04–5.32 | 0.039 |
| HPV 16/18 infection | 2.85 | 1.44–7.12 | 0.025 |
| p16 positive | 1.43 | 0.28–7.23 | 0.787 |
| Ki-67 positive | NC | | |
| >50% Ki-67-positive cells | 3.20 | 1.02–5.19 | 0.045 |
| **Multivariate** | | | |
| HSIL previous cytology | 2.55 | 1.04–6.29 | 0.042 |
| >50% Ki-67-positive cells | 2.68 | 1.07–6.72 | 0.035 |
| HPV16/18 | 2.50 | 0.96–6.56 | 0.062 |

CIN = cervical intraepithelial neoplasia, HSIL = high-grade squamous intraepithelial lesions, NC = could not be calculated.

*Adenocarcinoma in situ in 1 patient.

Variables found significant at p≤0.05 level in univariate analysis were considered for the multivariable analysis.

*Ki-67 cell expression over 50% independent of their distribution across the epithelium.
immunostaining is recommended for the differential diagnoses of lesions between precancer and low-grade.\textsuperscript{1} However, there is no data supporting the notion that CIN2 p16-positive lesions have a higher risk of underlying CIN3.

Regarding Ki-67 expression, Kruse et al evaluated 44 CIN1 and CIN2 biopsies, and they presented a Ki-67 progression risk model that assessed the stratification index and the percentage of Ki-67 positive cells in the middle-third layer of the epithelium to classify women into “low-risk” or “high-risk” progression categories. They concluded that quantitative Ki-67 analysis of CIN1 and CIN2 biopsies had a strong independent predictive value for progression.\textsuperscript{16} Their study required an interactive imaging analysis system for the measurements of several morphological parameters. In contrast, we try to define Ki-67 expression with 3 simple methods: categorizing as positive or negative, grading the epithelial expression by thirds, and calculating the percentage of Ki-67 cell expression independent of their distribution across the epithelium. Ki-67 cell expression over 50% in CIN2 biopsies was statistically significantly correlated with presence of CIN3 in cone results, although the difference is very close to nonsignificance (p = 0.043), and further studies are likely needed to confirm these data.

Concordant with previous pathology studies, when we graded the p16 and Ki-67 expressions in cone specimens by thirds, their epithelial distribution increased gradually according to the severity of CIN.\textsuperscript{14,15}

Excisional specimens with CIN3+ increase not only with the severity of the preceding histology, but also with the severity of the preceding cytology.\textsuperscript{25} We reported that most CIN3 cone results were statistically significantly associated with previous HSIL as compared with no HSIL previous cytology. Moreover, it is well known that HPV infection, especially HPV16/18, is the most important etiology of cervical cancer.\textsuperscript{26} A recent study that evaluated the effect of the specific genotypes on 416 patients treated with LEEP found that HPV16 and HPV18 were significantly more common in patients with CIN3+ than in those with CIN1/CIN2 (p<0.001).\textsuperscript{27} Interestingly, our univariate analysis supports these previous studies showing that HPV16/18 infection had an increased risk for CIN3.

One of the most important limitations of our study was that despite the fact that the CIN2 colposcopy-directed biopsy diagnosis was performed on the worst suspicious image at colposcopy, the biopsy still contains less index of the lesion than the subsequent cone specimen. Likewise, we could not define progression spontaneously to CIN3 for the short time between biopsy and cone results. However, we would like to evaluate the role of p16 and Ki-67 immunostaining in CIN2 biopsies to identify more aggressive occult lesions that might be treated by cone and not considered for expectant management. Ki-67 cell expression over 50% in CIN2 biopsies and HSIL in the previous cytology showed association to CIN3 cone results. Despite the fact that the results show statistically significant differences, these data should be considered with caution. Ki-67 cell expression over 50% was present in 65.7% of CIN2 biopsies where cone results showed CIN3 and 45.5% where cone results demonstrated ≤CIN2. Also, 61.8% of the women with a CIN3 cone result showed an HSIL on previous cytology, and 40.7% of the patients with ≤CIN2 in the cone specimen showed HSIL in the previous cytology. Following these recommendations, more than 40% of the women that might have been managed conservatively would be overtreated.

In conclusion, the current study supported the critical function of p16 and Ki-67 as a specific marker for accurate diagnosis of CIN grade; however, their usefulness for predicting underlying CIN3 in CIN2 biopsies is unclear. Patients with a Ki-67 cell expression over 50% in a CIN2 biopsy and HSIL in the previous cytology could have a higher risk of underlying CIN3, so they would be eligible to be treated by cone or more intensive management than would other patients with CIN2 who would benefit from conservative management. Further studies on the application of such p16 and Ki-67 biomarkers are needed to predict CIN2 evolution. To this end, we are conducting a prospective follow-up study to evaluate risk factors for progression (including p16 and Ki-67) in selected CIN2 patients.

References


