Assessment of Correlation Between p16\textsuperscript{INK4a} Staining, Specific Subtype of Human Papillomavirus, and Progression of LSIL/CIN1 Lesions

First Comparative Study

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Key Words: Low-grade squamous intraepithelial lesion; LSIL; Cervical intraepithelial neoplasia grade 1; CIN1; p16; Human papillomavirus; HPV subtype; Prognosis

ABSTRACT

Objectives: To study and compare the effectiveness of p16\textsuperscript{INK4a} staining and specific human papillomavirus (HPV) subtypes as a prognostic marker in cervical intraepithelial neoplasia grade 1 (CIN1; low-grade squamous intraepithelial lesions).

Methods: Sixty-four cervical samples diagnosed as CIN1 and stained with p16\textsuperscript{INK4a}, with HPV status assessed by polymerase chain reaction–direct sequencing.

Results: Of the 34 p16\textsuperscript{INK4a}-negative biopsy specimens, 26 regressed, seven persisted, and one progressed. Of the 20 p16\textsuperscript{INK4a} diffusely positive biopsy specimens, seven regressed, eight persisted, and five progressed. Ten biopsy specimens stained positive only in the lower one-third of the sample, of which seven regressed and three persisted. p16\textsuperscript{INK4a} diffusely positive CIN1 lesions were associated with only high-risk HPV subtypes, with the exception of one HPV-negative biopsy specimen. Three different high-risk HPV subtypes and one low-risk HPV subtype (HPV66) were identified in the six CIN1 lesions that progressed.

Conclusions: There is a significant relationship between p16\textsuperscript{INK4a} immunostaining and follow-up (\(P = .002\)). p16\textsuperscript{INK4a}-negative specimens or positivity in the lower one-third of CIN1 lesions seldom progress to a CIN2-3 lesion.
slight and mitotic figures are limited to the basal one-third of the epithelium.\textsuperscript{1} Approximately 11.4\% of women are infected by HPV at some point during their lifetime,\textsuperscript{1} and although almost all cervical carcinomas arise from CIN1,\textsuperscript{2} most of these CIN1 lesions are transient and regress spontaneously to normal epithelium.\textsuperscript{3-5} Still, 10\% to 15\% of these CIN1/LSIL lesions progress to CIN2-3/HSIL,\textsuperscript{6-10} which are considered the immediate precursors to cervical carcinoma.\textsuperscript{2} High-risk HPV (HR-HPV) is detected in approximately 85\% of these CIN1 lesions, thus providing little prognostic information to the diagnosis.\textsuperscript{11} As such, since the clinical and morphologic aspects of these lesions are not predictive of its evolution, there is a strong clinical interest in distinguishing the lesions with a higher chance of progression toward a CIN2-3/carcinoma, hence allowing a closer follow-up of the patients with a higher risk of progression and a less strict follow-up for the majority that most probably will normalize spontaneously.\textsuperscript{5,12}

The primary objective of this study was to confirm the effectiveness of the immunohistochemical marker p16\textsuperscript{INK4a}, presently used inconsistently as a diagnostic helper in CIN2-3 lesions, as an independent prognostic factor in CIN1. To our knowledge, this has been demonstrated in only three studies.\textsuperscript{13-15} A secondary objective, which was the first of its kind, was to identify among the HPVs with high oncogenic risk—those that would progress with near certainty from a CIN1 to a CIN2-3 lesion—and to subsequently correlate the p16\textsuperscript{INK4a} immunomarker with the specific subtype of HPV. An additional secondary objective was to then provide a test that would be reproducible and accessible even in remote areas, to distinguish the minority of patients with CIN1 lesions who are at higher risk of progressing toward a precancerous lesion.

**Materials and Methods**

Eighty-eight patients with an established diagnosis of CIN1 via a colposcopy-directed biopsy were randomly selected from the Sainte-Justine Hospital of Montreal, Québec, Canada. The biopsy specimens were then reviewed by two pathologists (D.B.-D.S. and M.R.) to confirm the diagnosis of CIN1. The biopsy specimens were fixed in 10\% buffered formalin, and paraffin-embedded, 3-\mu m-thick sections were stained with hematoxylin-phloxin-saffron. The diagnosis was established using exclusively morphologic criteria. Two parallel procedures were then performed for each specimen:

1. An immunostain with p16\textsuperscript{INK4a} was performed (Ventana immunostainer; Ventana Medical Systems, Tucson, AZ) following the recommendations of the supplier on the same paraffin block as the one from which the diagnosis was made. In each case, a positive control was included. A case was considered p16\textsuperscript{INK4a} positive when the marking was nuclear and cytoplasmic, with strong intensity, as well as localized in at least the lower one-third of the epithelium, following the conventions of other studies on this topic.\textsuperscript{16,17}

   The novel approach taken in this experiment is that cases in which the staining included the lower one-third but extended further through the surface epithelium (some with lower intensity than the basal one-third) were diagnosed as diffusely positive and hence distinguished from those with only positivity restrained to the lower one-third. This was done because previous studies were divided into two groups: those that considered p16\textsuperscript{INK4a} staining positive in at least the lower one-third\textsuperscript{13-18} and those that required it to be positive diffusely throughout the entire thickness of the epithelium.\textsuperscript{19} As a result, we decided to keep them separate and analyze the prognosis value of p16\textsuperscript{INK4a} for each group separately.

2. HPV typing was done using the polymerase chain reaction (PCR) sequencing from the DNA extracted from the same paraffin block. The biopsy specimens were fixed in formalin between 12 and 72 hours. The DNA extracted from these formalin-fixed biopsy specimens was processed with a standard PCR technique previously described and validated.\textsuperscript{20} More specifically, a two-tier PCR–direct sequencing (DS) approach based on the use of both MY09/MY11 and GP5+/GP6+ sets of primers was used. DS was done according to the manufacturer’s instruction using a BigDye Direct Cycle Sequencing Kit (Applied Biosystems, Melbourne, Australia). HPV sequences were identified in a papillomavirus database created in Microsoft Excel (Microsoft Corporation, Redmond, WA) and Corel Quattro Pro 9 (Corel Corporation, Ottawa, Ontario, Canada) formats. This sequence database is simultaneously a search-and-comparison tool for quick (several seconds) typing of HPV from regular and “degenerated” sequencing results. As a result, the exact subtype of HPV was obtained.

For all patients, the follow-up visit was scheduled at least 6 months following the date of the original biopsy. In the event that a follow-up biopsy had been performed prior to this 6-month threshold, an additional follow-up visit was scheduled adhering to the 6-month window after the biopsy, and the sample from that visit was used for the purpose of the study, as per the recommendations of the Society of Obstetricians and Gynaecologists of Canada.\textsuperscript{16} A few cases occurred in which the follow-ups were performed with endocervical curettage or a loop electrosurgical excision procedure (LEEP), and the resulting diagnosis was used. Furthermore, if a patient who had been diagnosed underwent LEEP with negative margins and was reinjected, she was considered a new patient.

The result of the follow-ups was categorized under three entities: `regression` was defined as the normalization of the epithelium; `persistence` was defined as the diagnosis of CIN1
(LSIL) on the follow-up, some of which ended up with a LEEP on the CIN1 lesion; and progression was defined as the development of a high-grade (CIN2-3/HSIL, carcinoma) lesion on follow-up.

Statistical Analysis

Statistical analysis was performed using SAS version 9.3 (SAS Institute, Cary, NC). All statistical tests were two-sided, and significance was set at $P < .05$. Fisher exact or $\chi^2$ tests were used for comparison. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated.

This project was evaluated and accepted by the ethical committee of Sainte-Justine Hospital.

Results

This study began with 88 patients of reproductive age (mean, 29.3 years) who had a cervical biopsy specimen that confirmed the CIN1 diagnosis. Nineteen patients were lost to follow-up, two had no residual lesion upon subsequent paraffin block cuts for the p16\textsuperscript{INK4a} immunostain, two had insufficient lesion or tissue for the subsequent PCR-HPV, and one had a confection and hence were excluded. Of the remaining 64, 40 (62.5%) regressed, 18 (28.1%) persisted, and six (9.4%) progressed. This concurs with the current data in the literature that estimates that 10% to 15% of CIN1 lesions evolve to high-grade lesions.\textsuperscript{6-10} In total, 14 (21.9%) patients had LR-HPV and 46 (71.9%) had HR-HPV; four (6.2%) concluded negative for HPV through PCR-DS.

On immunohistochemical staining Image 1 of the 64 samples, 34 (53.1%) were negative, 10 (15.6%) were positive in the lower one-third, and 20 (31.2%) were diffusely positive. From the 34 p16\textsuperscript{INK4a}-negative samples, 26 (76.5%) regressed, seven (20.6%) persisted, and only one (2.9%) progressed. On the other hand, from the 10 biopsy specimens with p16\textsuperscript{INK4a} positive in the lower one-third, seven (70.0%) regressed and the remaining three (30.0%) persisted. The follow-ups for the 20 samples in the p16\textsuperscript{INK4a} diffusely positive group were closely divided to seven, eight, and five for regression, persistence, and progression, respectively. Table 1 summarizes these results.

The 64 paraffin blocks, from the same sample of the biopsy specimen on which the diagnosis was made, were then available for the HPV PCR-DS. Four (6.2%) were shown to be negative for HPV using the PCR-DS method, of which one progressed to a high-grade lesion, one persisted, and two regressed. In the 14 (21.9%) specimens with the LR-HPV subtype, one HPV66 progressed, four persisted, and nine regressed. In the 46 (71.9%) specimens with the HR-HPV subtype, four progressed, 13 persisted, and 29 regressed. Table 2 summarizes these results.

Furthermore, from the 34 (53.1%) p16\textsuperscript{INK4a}-negative biopsy specimens, 19 (55.9%) were HR-HPV, 12 (35.3%) were LR-HPV, and one (8.8%) was negative. Similarly, for the 10 p16\textsuperscript{INK4a}-positive samples in the lower one-third, eight were high risk and two were low risk. For the 20 (31.2%) p16\textsuperscript{INK4a} diffusely positive cases, 19 were associated with HR-HPV and one with negative HPV.

In the six (9.4%) CIN1 lesions that progressed to CIN2-3, there were three different types of HR-HPV (more specifically, HPV16 twice, HPV58, and HPV82), one low-risk HPV66, and one negative for HPV; the latter is noteworthy to mention since it was p16\textsuperscript{INK4a} diffusely positive. Figure 1 summarizes the HPV subtypes and details the p16\textsuperscript{INK4a} staining associated with each entry and its respective follow-up.

Discussion

There were three significant observations from our study. First, our study confirms the value of p16\textsuperscript{INK4a} as a prognostic marker for LSIL/CIN1 lesions. This is in accordance with the three previously published studies that have shown that p16\textsuperscript{INK4a}-negative expression is associated with lower rates of progression toward an HSIL/CIN2-3.\textsuperscript{13-15} The subdivision of p16\textsuperscript{INK4a} reading into negative, positive lower one-third, and positive diffusely throughout all layers yielded interesting information. The results showed that from the 34 p16\textsuperscript{INK4a}-negative CIN1 lesions, only one progressed, while in the 10 p16\textsuperscript{INK4a} samples with lower one-third positivity, none progressed. Given this, we hypothesize that completely

<p>||p16 Immunostaining vs Follow-up Status||</p>
<table>
<thead>
<tr>
<th>No. of Samples</th>
<th>p16 Immunostaining</th>
<th>Regressed</th>
<th>Persisted</th>
<th>Progressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16 negative</td>
<td>26</td>
<td>7</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>p16 positive in lower one-third</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>p16 diffusely positive</td>
<td>7</td>
<td>8</td>
<td>5</td>
<td></td>
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<p>||HPV Type vs Follow-up Status||</p>
<table>
<thead>
<tr>
<th>No. of Samples</th>
<th>Type</th>
<th>Regressed</th>
<th>Persisted</th>
<th>Progressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV negative</td>
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<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LR-HPV</td>
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<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HR-HPV</td>
<td>29</td>
<td>13</td>
<td>4</td>
<td></td>
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HPV, human papillomavirus; HR-HPV, high-risk HPV; LR-HPV, low-risk HPV.
Image A and B, Cervical intraepithelial neoplasia grade 1 (CIN1) lesion with its associated $p16^{INK4a}$-negative staining. C and D, CIN1 lesion with its associated $p16^{INK4a}$-positive staining in the lower one-third. E and F, CIN1 lesion with its associated $p16^{INK4a}$ diffusely positive staining.
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negative p16\textsuperscript{INK4a} and those that stain only in the lower one-third layer of the epithelium seldom progress to a high-grade lesion. In our pilot study, p16\textsuperscript{INK4a} has a clinically useful sensitivity of 83% with a negative predictive value of 97.1%. Interestingly, the one CIN1 p16\textsuperscript{INK4a}-negative lesion that progressed was associated with a low-risk HPV66, and hence an HPV test would not have provided any additional warning to the clinician. On the other hand, of the 20 diffusely positive p16\textsuperscript{INK4a} CIN1 lesions, the follow-ups were distributed uniformly into the three categories of regression, persistence, and progression, resulting in a lower specificity of 57%.

As a result, we can draw the conclusion that there is a significant relationship between p16\textsuperscript{INK4a} and the follow-up (£P = .002£); when p16\textsuperscript{INK4a} is negative or with a positivity restricted to the lower one-third of the epithelium of a CIN1 lesion, it seldom progresses to a CIN2-3/carcinoma lesion. p16\textsuperscript{INK4a} is thus a very valuable and clinically sensitive test.

Second, this study is the first of its kind to correlate CIN1 lesions with their exact subtypes of HPV using PCR-DS. This allows a direct study on each specific HPV subtype and its follow-up, as well as a possible relationship with the associated p16\textsuperscript{INK4a}. A total of 14 LR-HPV specimens were detected, of which only one HPV66 progressed to a high-grade lesion, requiring a LEEP therapy; two other HPV66 lesions were also detected, both of which regressed back to normal. All three of these HPV66 samples were p16\textsuperscript{INK4a}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Human papillomavirus (HPV) subtype correlated with p16 expression and follow-up.}
\end{figure}
negative. On the other hand, in the HR-HPV group, four (8.7%) of the 46 lesions progressed to a high-grade lesion, none of which was $p16^{INK4a}$ negative or even positive in the lower one-third. Although all four cases were $p16^{INK4a}$ diffusely positive, this yields a lower correlation since the $p16^{INK4a}$ was also diffusely positive in eight (61.5%) of 13 HR-HPV lesions that persisted, as well as seven (24.1%) of 29 HR-HPV lesions that regressed back to normal. Similarly, for the only lesion that progressed without an identifiable HPV subtype, the $p16^{INK4a}$ was neither negative nor limited just to the lower one-third.

In our study, no association was found between the specific subtype of HPV and the follow-up ($P = .83$). Moreover, among the six CIN1 lesions that progressed toward a high-grade lesion in our cohort, four were HR-HPV (HPV16 twice, HPV58, and HPV82), all with a diffusely positive $p16^{INK4a}$; one was an LR-HPV (HPV66) with a completely negative p16 marking; and one was a negative HPV but marked diffusely with $p16^{INK4a}$.

In addition, although not common, there was one case of coinfection in a 30-year-old woman with two HPVs (HPV16 and HPV82). Although both were HR-HPVs, they were $p16^{INK4a}$ negative, and the lesions ended up regressing back to normal in the follow-up; we eliminated them from the data to avoid confusion.

Upon combining the HPV subtype with the $p16^{INK4a}$ staining, we can notice that with the exception of one HPV-negative specimen, all of the 20 $p16^{INK4a}$ diffusely positive cases were associated with HR-HPV. For those that were $p16^{INK4a}$ positive in the lower one-third, with the exception of two LR-HPVs, the other eight were HR-HPVs. This is in contrast with the $p16^{INK4a}$-negative group, which had an almost random distribution between 19 HR-HPVs and 12 LR-HPVs, plus the three negative HPVs.

Throughout the course of the work captured in this study, we were aware of the possible disadvantage of our study design, in which the HPV subtype was not reevaluated on follow-up specimens. However, this was considered acceptable given that a secondary goal of the study was to determine whether the analysis of $p16^{INK4a}$ would be suitable for a standard laboratory performing routine work within typical limitations. Furthermore, as the study suggested, our hypothesis is that it is not the HPV subtype to which an LSIL/CIN1 lesion is associated that provides insight but rather the $p16^{INK4a}$ expression within the lesion.

**Conclusions**

Several previously published studies considered that the expression of $p16^{INK4a}$ restricted to the lower one-third is positive. Our data suggest that those confined only to the lower one-third should be grouped with the negatives, and true positivity, from a prognostic point of view, should be diagnosed when the cervical epithelium expresses $p16^{INK4a}$ diffusely, even when the staining is less intense in the more superficial layers. Further studies with larger cohorts are encouraged.

Although there is some correlation between the $p16^{INK4a}$ positive and the HR-HPV lesions, this is of no clinical use since the HPV subtype is a poor predictor of the behavior of a CIN1 lesion. On the other hand, in our relatively small patient sample, $p16^{INK4a}$ staining proved to be an excellent and sensitive test that would help in selecting patients who require a less intensive follow-up. Specifically, patients with a CIN1 lesion that is negatively stained or has positivity confined to the lower one-third seldom progress to high-grade precancerous lesions. In our study, 44 of the 64 patients would not have required a close follow-up.

Further studies with larger cohorts are encouraged to validate our hypothesis. If our hypothesis is validated, it would justify using $p16^{INK4a}$ immunostaining as a simple solution that is highly reliable and reproducible for identifying patients with CIN1/LSIL at risk of developing precancerous lesions, even in remote areas where more sophisticated diagnostic modalities are not available.

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**References**


