

Interpretation of p16 Immunohistochemistry In Lower Anogenital Tract Neoplasia

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Background

p16^{INK4A}

- p16^{INK4A} (henceforth referred to as p16) immunohistochemistry (IHC) is a good surrogate test for the presence of a potentially transforming human papillomavirus (HPV) infection in anogenital carcinomas and premalignant lesions¹.
- p16 is a 16 kDa protein encoded by *CDKN2A*, within the INK4/ARF tumour suppressor locus on Chromosome 9 (9p21.3)^{2,3}.
- Its major function in the cell is to inhibit cyclin-dependent kinases (CDK4 and CDK6) that are required to phosphorylate the retinoblastoma protein, pRb. In this way it inhibits traversal of the G1/S checkpoint, resulting in blockade of the cell cycle.
- It is therefore a marker of cellular senescence, or expressed in response to aging or other stressors, to curb inappropriate cell division.
- Its role in cancer is complex; as a tumour suppressor its function is deranged in a large variety of cancers, and it is a commonly mutated gene in cancer, both at the outset and during progression.
- p16 is INACTIVATED in about 50% of all human cancers by a variety of mechanisms, most commonly homozygous deletion of the gene.
- p16 OVEREXPRESSION, as occurs in HPV-driven cancers, occurs as a result of its increased production due to transcriptional release from negative feedback control, as discussed below.

p16 in high-risk HPV infection

- Infection with high-risk HPV (hrHPV) is an early event in the multistep process of carcinogenesis in various organs (for example cervix, vagina, vulva, anal canal, head and neck)^{4,5}.

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- hrHPV infection can result in a spectrum of changes from silent/asymptomatic infection, in which the virus remains in latent form in basal cells and there is no resulting clinically or morphologically apparent lesion, through low grade lesions (low-grade squamous intraepithelial lesion (LSIL); koilocytosis/CIN1 and equivalent lesions in other organs) to high grade lesions (high-grade squamous intraepithelial lesion (HSIL); CIN2/CIN3 and equivalent lesions in other organs) and carcinoma.
- The ability of hrHPVs to drive neoplastic change reflects differences in the functions and cellular effects of their protein products, principally E6 and E7.
- In low-risk productive HPV infections, viral E6 and E7 expression is tightly regulated and the primary role of these proteins is to drive cell cycle entry in terminally differentiated suprabasal cells, promoting viral genome amplification in these layers.
- In hrHPV infections, E6 and E7 proteins are expressed in the basal layers where they result in cell proliferation and evasion of host immune surveillance.
- Persistence of hrHPV infection over time results in deregulation and thereby increase of E6/E7 expression which is necessary for neoplastic transformation; while expression can be deregulated with the virus in episomal form, this becomes much more profound when the virus is integrated and the E6/E7 oncogenes are released from transcriptional control due to disruption of the viral E2 region.
- E6 and E7 have the ability to bind and inactivate p53 and pRb respectively; this promotes rapid progression through the cell cycle without p53-mediated control of DNA integrity.
- The binding and degradation of pRb, and related proteins p107 and p130, releases p16 production from its negative feedback control, with a paradoxical increase in levels of the protein in the cell; this represents an attempt by the cell to inhibit the uncontrolled proliferation⁶.
- Abnormal over expression of p16 is therefore a marker of E7-mediated functional inactivation of pRb and may be used as a surrogate for a hrHPV infection with potential to transform.⁷
- Deregulated E6/E7 expression is necessary but not sufficient for transformation, which results from the accumulation of additional genomic abnormalities: in addition to viral persistence, host immune response and viral integration, many other factors influence progression such as the nature of

the target tissue (junctional vs non-junctional epithelium), hormonal factors and the presence of co-factors or synergists, eg smoking.⁴

p16 overexpression in anogenital neoplasia

- Immunostaining for p16 has emerged as a surrogate for detection of potentially transforming hrHPV infection.^{1,8}
- The establishment of clear criteria for reporting a p16 immunostaining result has occurred gradually, analogous to establishing a diagnostic cut-off for interpretation of *HER2* immunostaining in breast carcinoma more than a decade ago, in that low levels of p16 immunoreactivity do not correlate with transforming hrHPV infection, just as low levels of *HER2* immunostaining do not correlate with *HER2* amplification or response to targeted therapy with trastuzumab in breast carcinoma.
- Progress towards an optimal cut-off has arguably proceeded more rapidly for head and neck squamous cell carcinoma, where HPV status, as determined by p16 immunostaining, has emerged as an important prognostic indicator, and is also predictive of response to radiotherapy.⁹⁻¹¹
- HPV-related vulval squamous cell carcinoma (VSCC) has similarly been shown to have a superior clinical outcome and response to adjuvant treatment as compared with non-HPV related VSCC, and p16, as a reliable marker of HPV-driven VSCC, therefore has prognostic significance.^{12,13}
- In precancerous squamous lesions, p16 overexpression signifies a potentially transforming hrHPV infection; p16 expression should not be used as a surrogate for grading these lesions and grading should be based on morphological criteria. However, p16 may be useful in distinguishing high-grade lesions from benign lesions which may mimic these (see later).
- Lesions associated with low-risk HPV infection DO NOT show block-type p16 positivity. They may however show patchy non-block-type staining.
- The use of p16 IHC improves diagnostic agreement in cervical biopsy interpretation⁷.
- Although the results of studies are contradictory, abnormal p16 immunohistochemistry does not reliably predict risk of progression of low grade cervical squamous lesions.
- Precancerous hrHPV related cervical glandular lesions are almost invariably p16 positive.

- The Lower Anogenital Squamous Terminology (LAST) consensus group have put forward guidance for the use of p16, as detailed below.

p16 immunohistochemistry interpretation

Normal/reactive expression patterns in squamous epithelium (Figure 1)

- Normal squamous epithelium generally shows completely absent expression.
- Occasional scattered, weakly staining cells may be seen, especially in immature metaplastic epithelium.
- In reactive conditions positively stained cells may be more numerous and/or stronger staining; these will still be irregularly scattered.

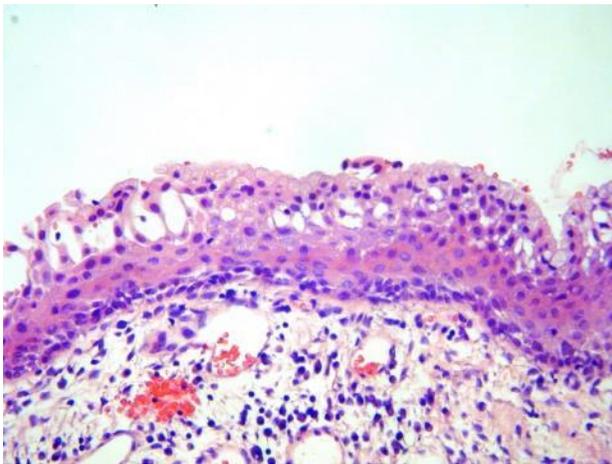


Figure 1a. Immature squamous metaplasia of the cervix.

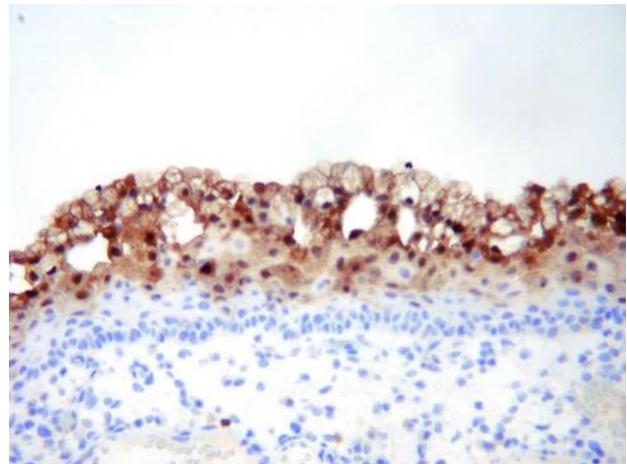


Figure 1b. p16 staining in the immature metaplastic squamous epithelium is typically patchy *with sparing of the basal layer*.

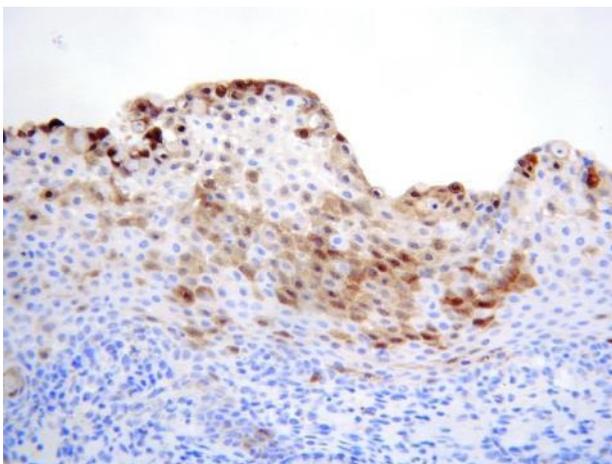


Figure 1c. Patchy p16 staining in normal cervical squamous epithelium.

Normal/reactive expression patterns in glandular epithelium (Figure 2)

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- Normal endocervical epithelium usually shows completely negative or absent staining.
- In reactive epithelium occasional positively stained cells may be observed; these are typically weakly stained and scattered irregularly within the epithelium.
- Tubo-endometrial metaplasia and lower uterine segment endometrial epithelium generally show patchy staining of epithelial cells.

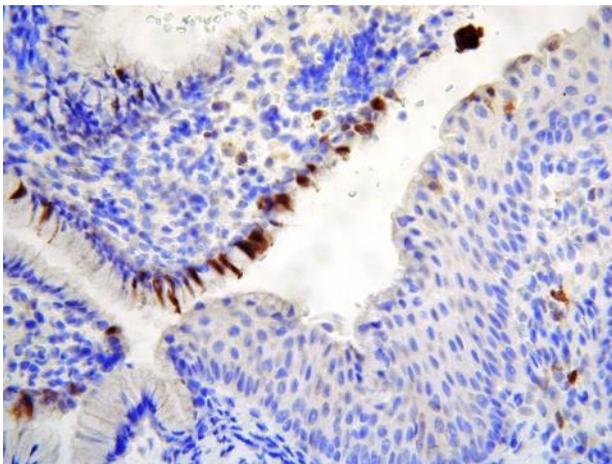


Figure 2a. Normal endocervical glandular epithelium may occasionally exhibit patchy p16 staining.

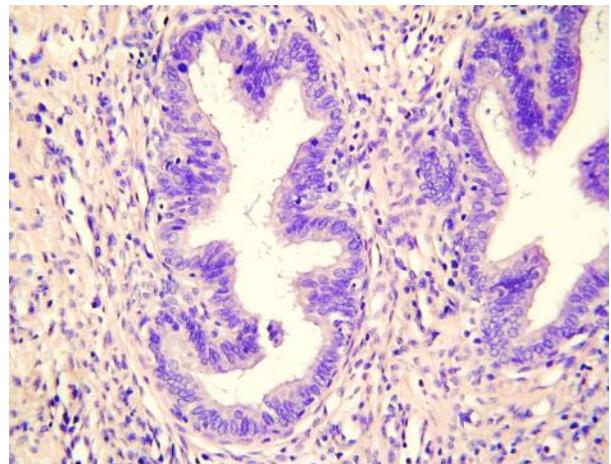


Figure 2b. Tuboendometrial metaplasia of the cervix can sometimes mimic adenocarcinoma in-situ with the nuclear pseudostratification and mitotic activity.

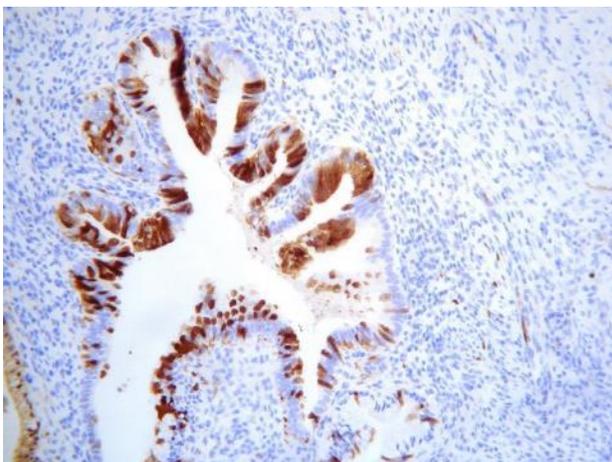


Figure 2c. Patchy p16 staining with mosaic pattern is typically found in tuboendometrial metaplasia of the cervix.

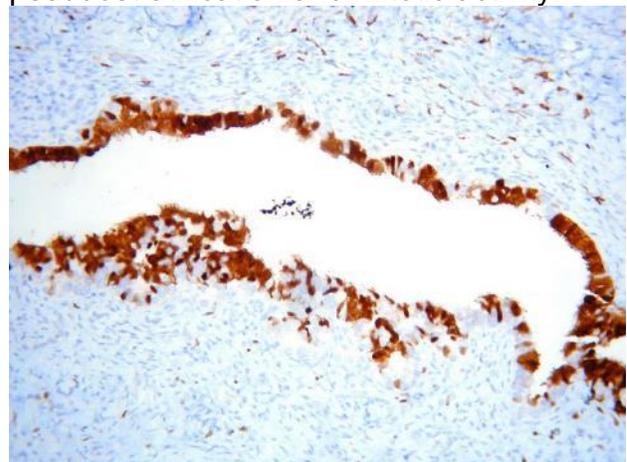


Figure 2d. The p16 staining in tuboendometrial metaplasia is sometimes quite extensive, although usually with

scattered negative staining cells. The pattern of staining may be mistaken as diffuse positive when the gland is partially sampled or encountered in a small biopsy.

Abnormal expression in squamous epithelium (Figures 3-7)

- **Abnormal expression in squamous epithelial lesions fulfils ALL of the following (this pattern has been described as *BLOCK POSITIVE* staining):**
 - Strong and continuous nuclear OR more typically nuclear and cytoplasmic expression in all epithelial cells in the basal and parabasal layers with upward extension
 - Upward extension must involve at least the lower one-third of the epithelial thickness
 - Abnormal expression must extend for at least 6 cells across
 - It is acknowledged that the criteria defining the horizontal and upward extent are arbitrary but these serve to improve specificity
- **Reporting terminology:**
 - Use of the word 'positive' is not recommended in pathology reports owing to potential for confusion.
 - Report as
 - PRESENCE vs ABSENCE OF ABNORMAL (DIFFUSE/BLOCK POSITIVE) EXPRESSION
 - OR
 - ABNORMAL (DIFFUSE/BLOCK POSITIVE EXPRESSION) vs NEGATIVE/NORMAL expression

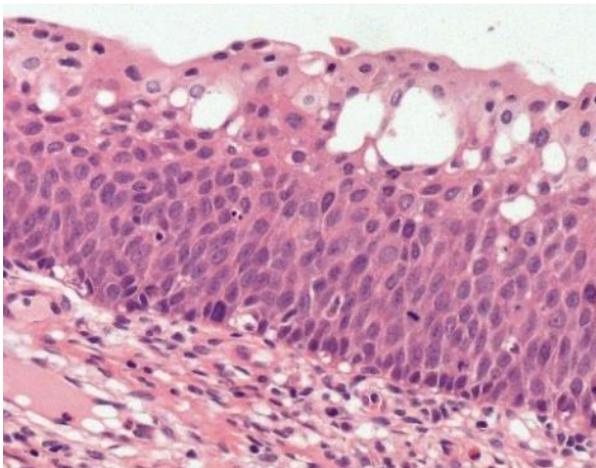


Figure 3a. HSIL (CIN2) of the cervix.

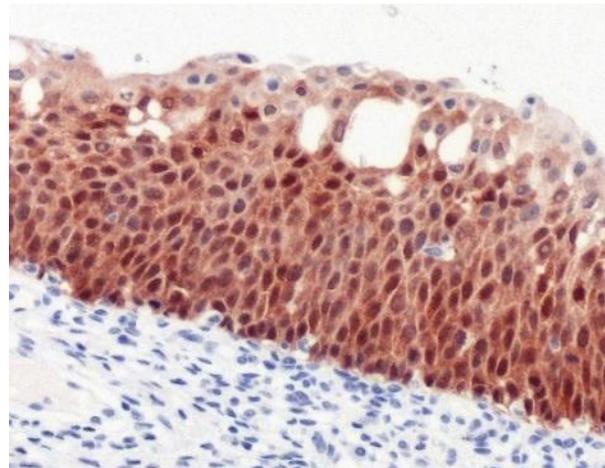


Figure 3b. Diffuse/block positive expression of p16 in HSIL.

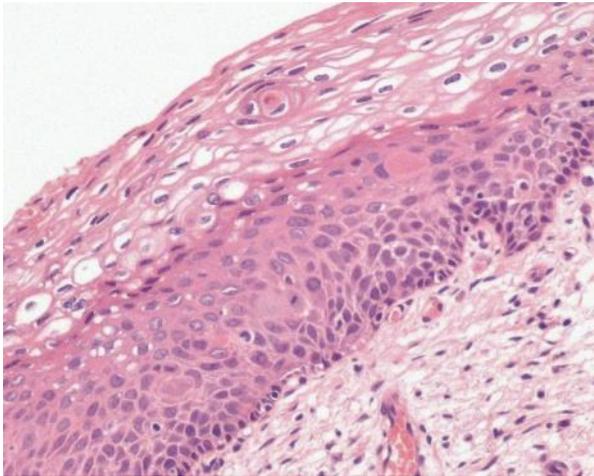


Figure 4a. Cervical squamous epithelium with morphological features indeterminate between HSIL and LSIL.

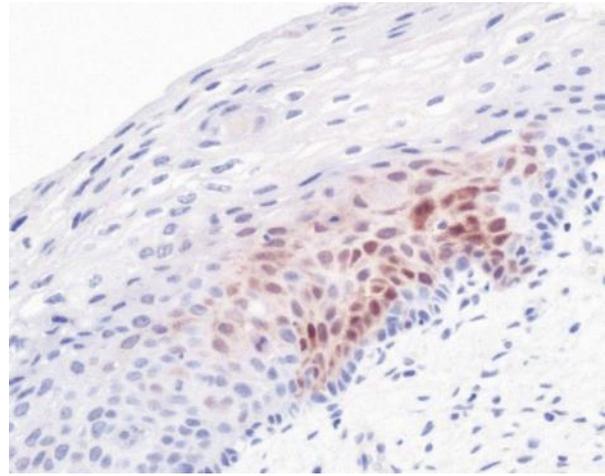


Figure 4b. Patchy non-block staining for p16 supports the diagnosis of a non-hrHPV-associated LSIL in this context.

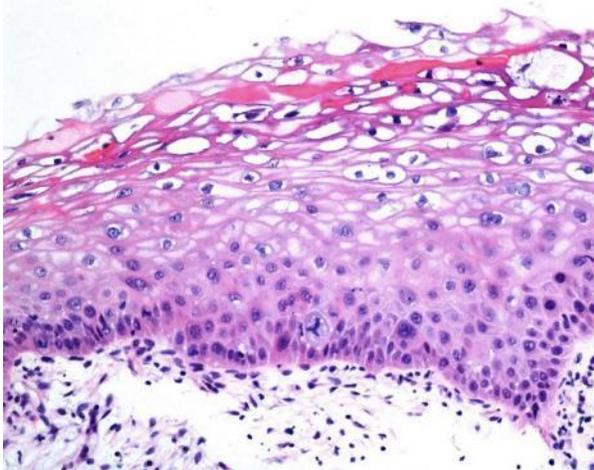


Figure 5a. Cervical squamous epithelium with maturation pattern resembling LSIL but atypical mitotic figure concerning for HSIL.

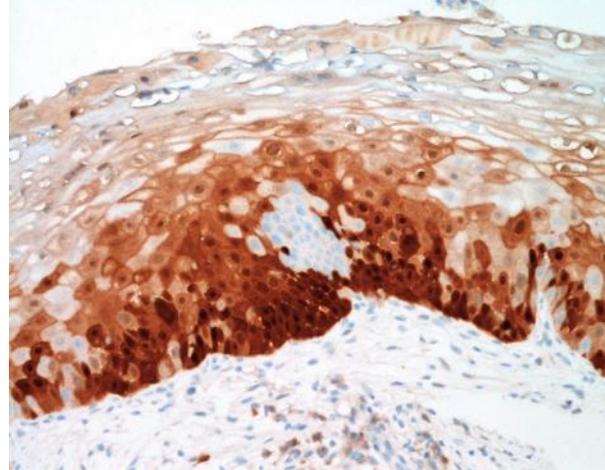


Figure 5b. The p16 staining meets the criteria of continuous basal positivity with upward extension to the lower one-third of the epithelial thickness, qualifying as diffuse/block positive. Note that this does not determine lesion grade, which should be assessed morphologically.

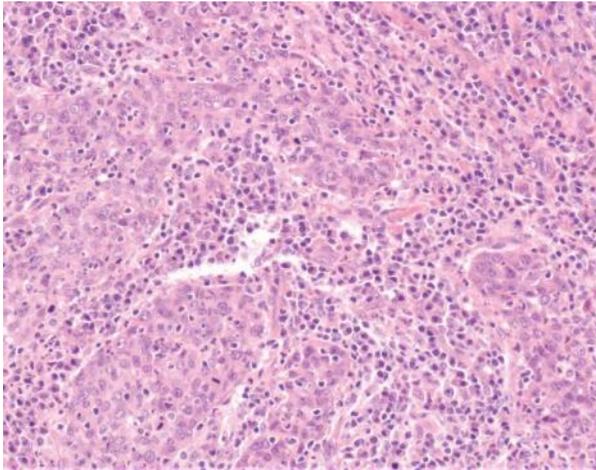


Figure 6a. Cervical squamous cell carcinoma, non-keratinizing type.

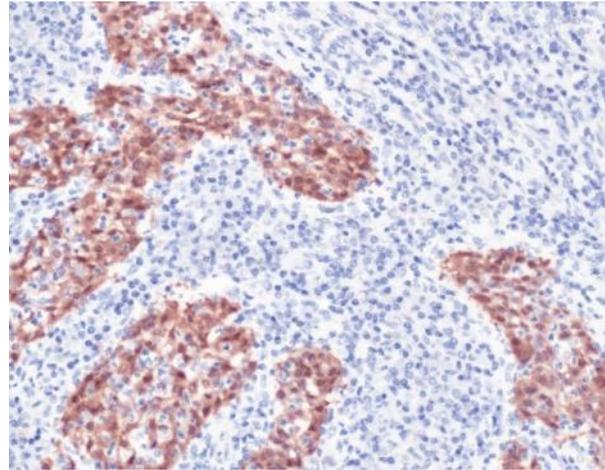


Figure 6b. Diffuse p16 staining in the carcinoma cells are typical of hrHPV-associated carcinoma.

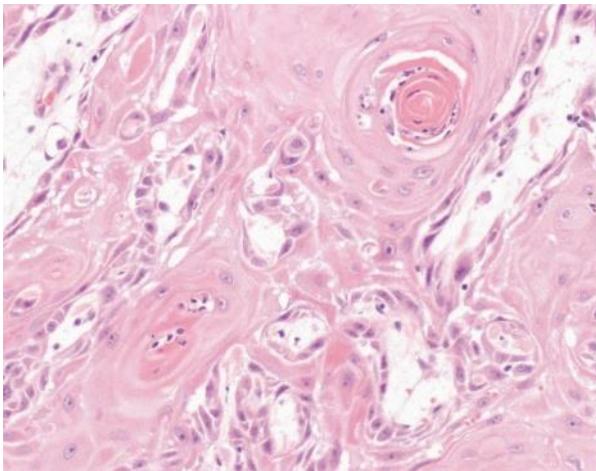


Figure 7a. Vulval squamous cell carcinoma, keratinizing type.

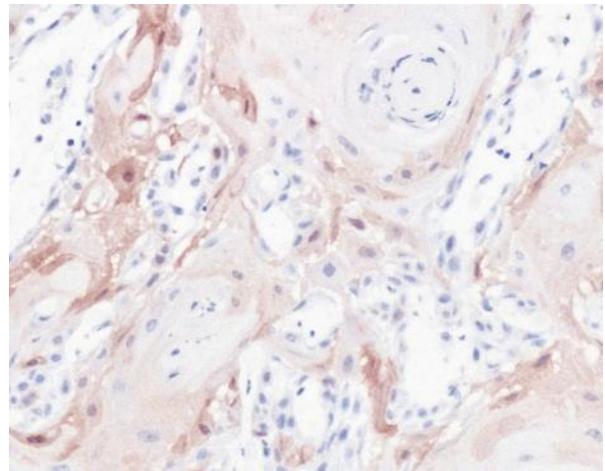


Figure 7b. Focal non-block p16 staining in the absence of continuous basal positivity is in keeping with an HPV-independent aetiology.

Abnormal expression in glandular epithelium (Figures 8-10)

- **Abnormal expression in glandular epithelial lesions is strong and continuous DIFFUSE positive staining** in glandular epithelial cells; staining may be nuclear or more commonly nuclear and cytoplasmic.
 - This is a sensitive and specific diagnostic marker of a transforming hrHPV infection *in the correct context*.
 - **Reporting terminology:**
 - Use of the word 'positive' is not recommended in pathology reports owing to potential for confusion.
 - Do not use the term 'block-type' for glandular lesions as this term relates specifically to squamous lesions
 - Report as PRESENCE vs ABSENCE OF ABNORMAL DIFFUSE POSITIVE
- OR
- ABNORMAL DIFFUSE POSITIVE vs NEGATIVE/NORMAL/PATCHY expression

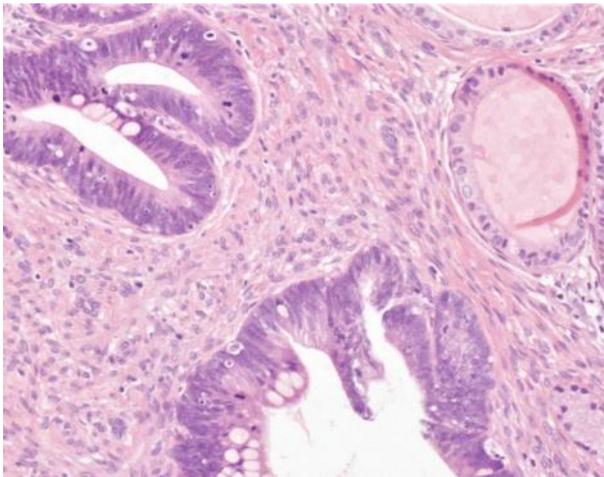


Figure 8a. Adenocarcinoma in-situ (AIS) of the cervix (high-grade cervical glandular intraepithelial neoplasia; CGIN).

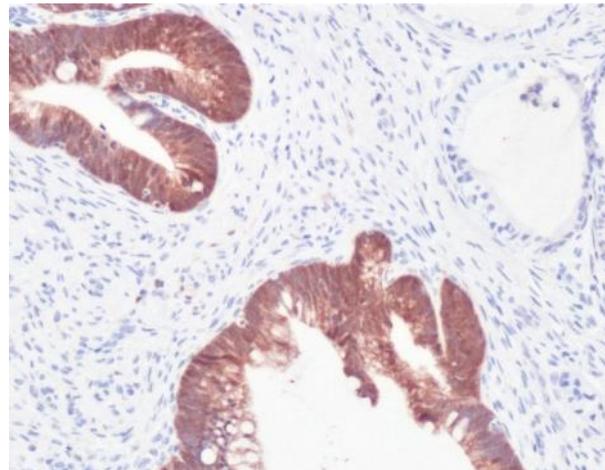


Figure 8b. Diffuse strong staining for p16 is present in the neoplastic glands, contrasting with the adjacent negative normal gland.

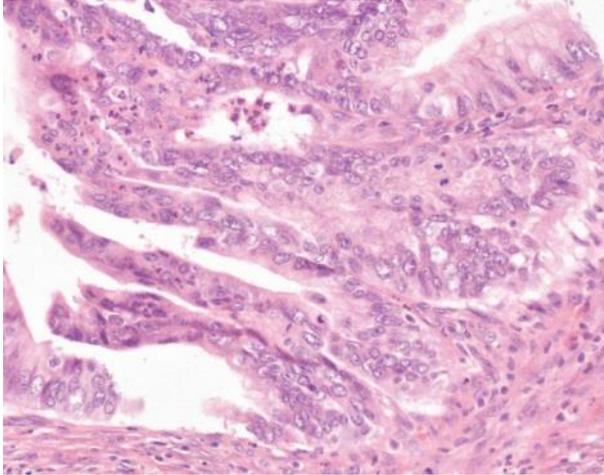


Figure 9a. Usual endocervical adenocarcinoma of the cervix.

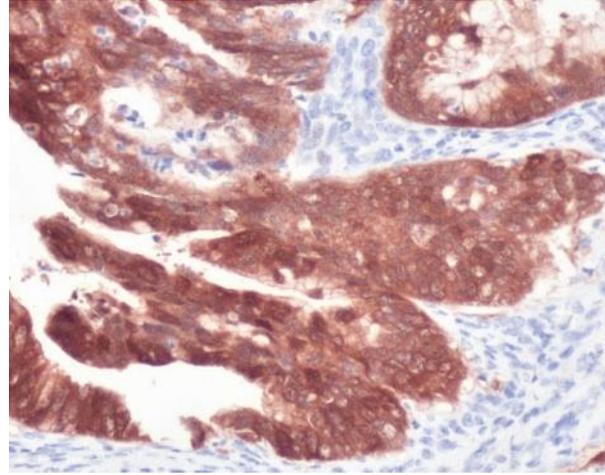


Figure 9b. Typical diffuse positive p16 staining is seen in this hrHPV-associated adenocarcinoma.

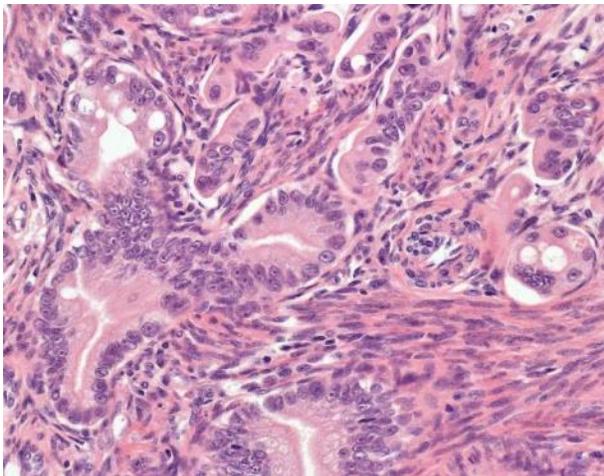


Figure 10a. Gastric-type adenocarcinoma of the cervix.

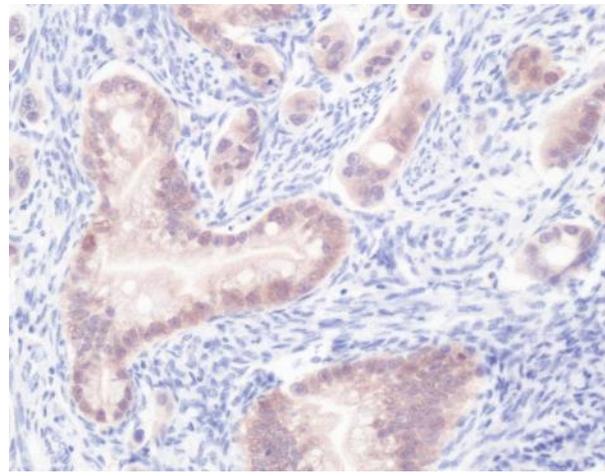


Figure 10b. Patchy weak p16 staining reflects the HPV-independent nature of this tumour.

Significance of p16 in anogenital intraepithelial neoplasia

Can p16 be used to grade squamous intraepithelial neoplasia?

- p16 is not a surrogate for grade; up to 50% of cases of LSIL (HPV/CIN1) are p16 positive.
- Grading of SIL (CIN) should be based on morphological criteria.

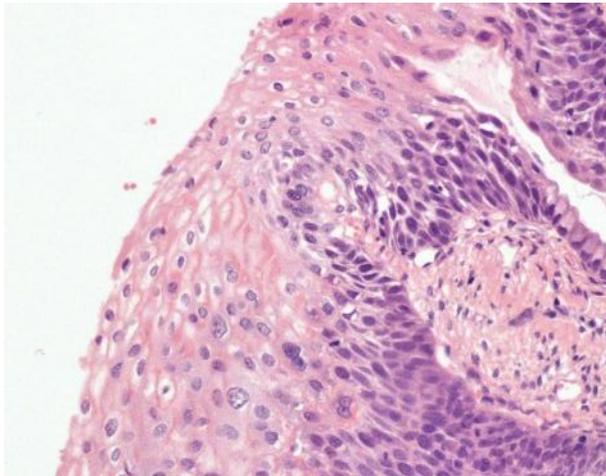


Figure 11a. LSIL (CIN1) of the cervix.

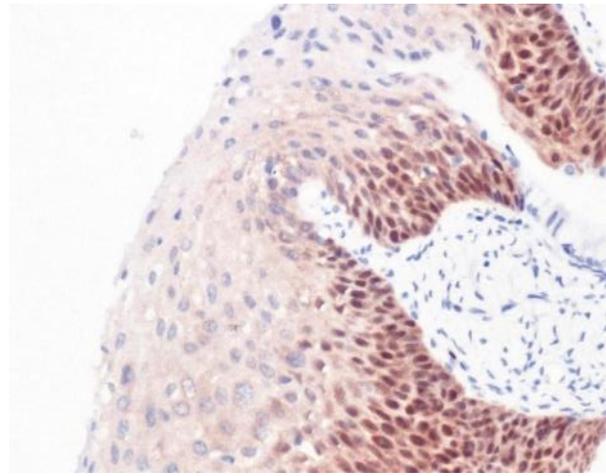


Figure 11b. Block positive p16 staining may be observed in LSIL (CIN1).

Does the thickness of stained epithelium matter?

- Although staining broadly mirrors the extent of epithelial differentiation, the extent of vertical p16 staining within the squamous epithelium does not *necessarily* correlate with CIN grade.

Is p16 expression abnormal in all high risk HPV infections?

- Abnormal p16 IHC is a marker of E7 mediated inactivation of Rb protein and is therefore a *diagnostic* marker of transforming hrHPV infection (*necessary but not sufficient* for progression to cancer).
- p16 expression is not abnormal in ALL hrHPV infections as these form a spectrum from silent, though productive to transforming/abortive infections; abnormal p16 expression is not seen in silent/latent infections and in half of the CIN1 lesions caused by hrHPV.

Is abnormal p16 expression diagnostic of high grade CIN?

- As stated above, up to 50% of LSIL (HPV/CIN1) is also p16 positive; p16 immunohistochemistry does not replace conventional grading.

Does abnormal p16 expression indicate a likelihood of progression to cancer?

- There are many conflicting results in published studies but, overall, based on the results seen in adequately powered studies, p16 can NOT be used as a reliable marker of increased risk of progression of CIN1.
- >90% CIN1 and up to 2/3rds CIN2 in young women will spontaneously regress after biopsy.

Can p16 expression be normal in high-grade squamous intraepithelial neoplasia, high-grade cervical glandular intraepithelial neoplasia (adenocarcinoma in situ-AIS) and HPV-related carcinoma?

- Rarely there may be complete absence of staining due to technical reasons; inactivation of the p16 gene through gene deletion or epigenetic silencing occurs in a variety of cancers and could theoretically occur during progression of HPV-mediated cancers and pre-cancers.¹⁴
- AIS of gastric-type rarely occurs in the cervix and p16 expression is normal.¹⁵

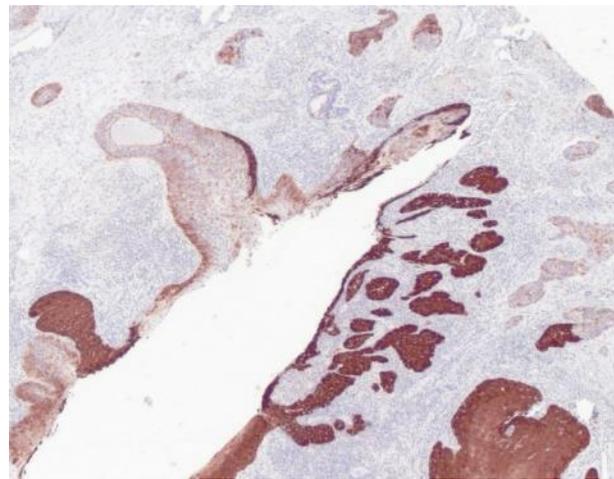
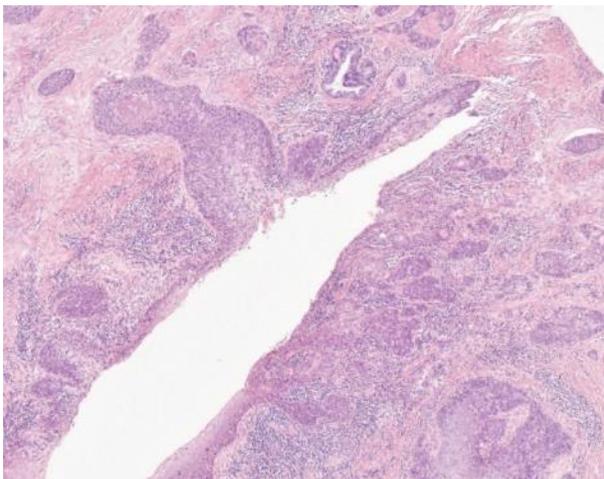


Figure 12a. Vulval HSIL (usual VIN) and squamous cell carcinoma. This case was confirmed as HPV-associated by in-situ hybridization (not shown), but demonstrates unusual pattern of p16 staining.

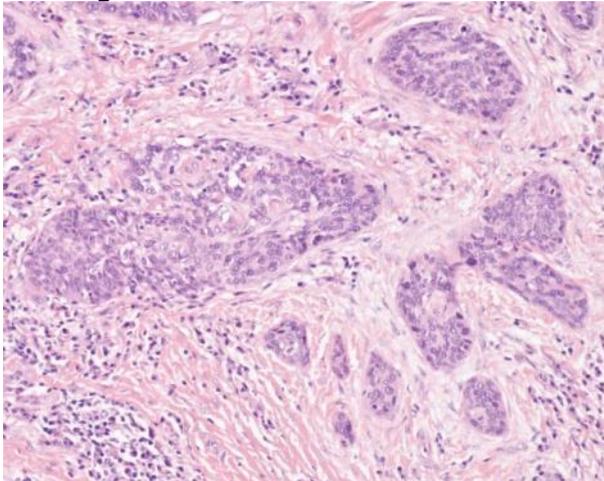


Figure 12c. High power view of the invasive squamous cell carcinoma (same case as Figure 12a).

Figure 12b. Diffuse/block p16 staining is observed in most areas of the HSIL with patchy absence of staining. The invasive component also demonstrates patchy absence of p16 staining.

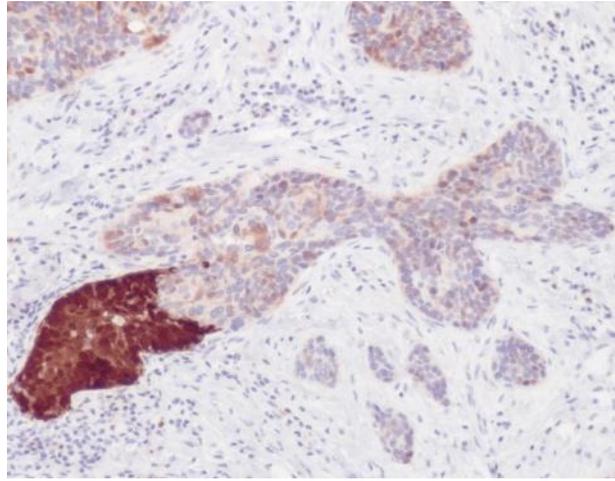


Figure 12d. Patchy weak p16 staining is observed in some areas of the invasive carcinoma, coexisting with foci of block positivity.

Can p16 be abnormally expressed in non-HPV related precancerous and cancerous lesions?

- p16 overexpression (including diffuse immunoreactivity) occurs in some non-HPV-related cancers of the female genital tract such as high grade tubo-ovarian serous carcinoma, uterine serous carcinoma and leiomyosarcoma. In addition, other tumour types, such as uterine and ovarian endometrioid adenocarcinomas, may exhibit significant staining, although this is usually patchy (non block-type). The interpretation of p16 staining is therefore context-dependent, as in these instances overexpression is likely to be a result of abrogation of the pRb pathway by mechanisms other than viral oncoprotein-mediated degradation, including gene mutation or deletion
- By the same token p16 overexpression may be occasionally seen in non-HPV related cervical gastric type adenocarcinomas, as well as occasional (2-3%) HPV-independent vulval squamous cell carcinomas

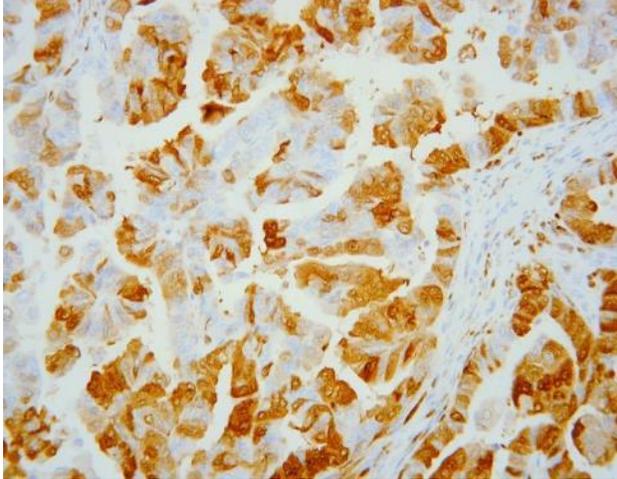


Figure 13a. Endometrioid adenocarcinoma. Mosaic pattern of p16 staining is typically seen.

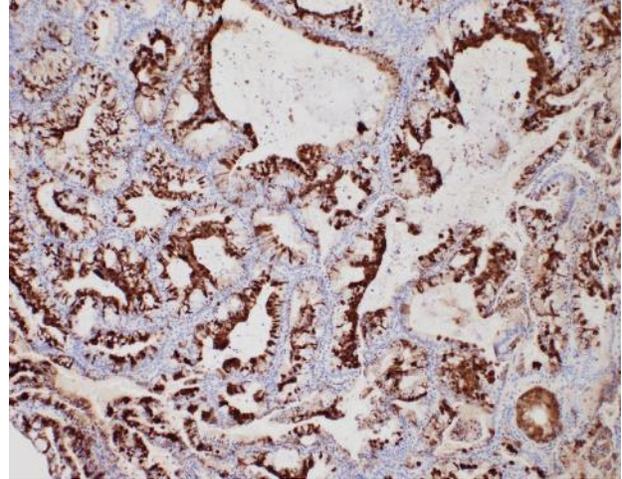


Figure 13b. Some cases of endometrioid adenocarcinoma may demonstrate nearly diffuse staining although scattered negative cells are usually observed.

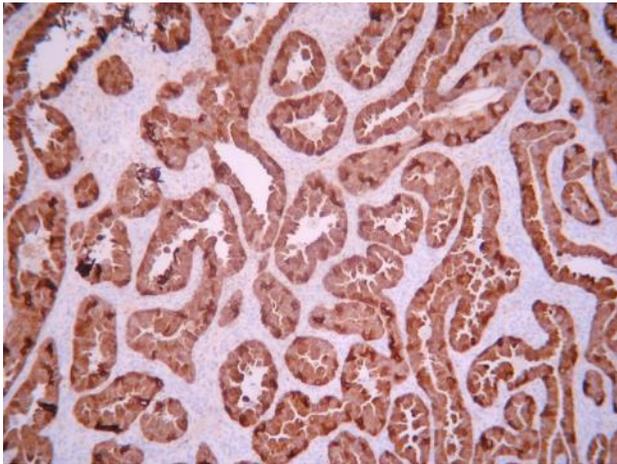


Figure 14a. High grade serous carcinoma of tubo-ovarian origin often demonstrate diffuse positive staining for p16, via mechanisms unrelated to hrHPV.

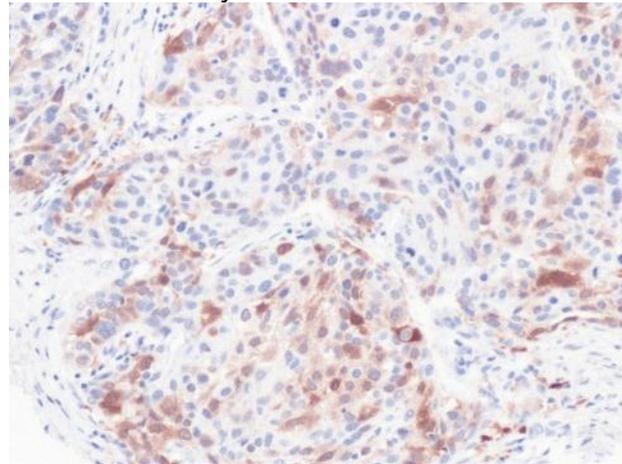


Figure 14b. Patchy weak p16 staining may also be seen in some cases of tubo-ovarian high grade serous carcinoma.

Indications for adjunctive p16 staining (based on the LAST criteria, Working Group 4: Biomarkers in HPV-Associated Lower Anogenital Squamous Lesions)

General points:

- Evidence for recommending usage of biomarkers in routine practice was ONLY available for p16 at the time of writing of these guidelines.
- p16 recommendations are applicable to HPV-related squamous lesions at all sites.
- Strong and diffuse block-positive p16 results support a categorization of precancerous disease.
- ***Any identified p16-positive area must meet H&E morphological criteria for a high-grade lesion to be interpreted as such.***
- Current evidence does not support any combination of markers to substantially improve performance when compared with the use of p16 alone.
 - p16 + Ki67: The overall improvement of performance (sensitivity and specificity) was minimal when compared with the p16 result alone; hence, the routine addition of Ki-67 to p16 IHC is not recommended.
 - ProExC +/- p16: ProExC performs in a similar manner to p16; at the time of writing, there was insufficient evidence to make an independent recommendation for use.
 - In cases for which p16 IHC is inconclusive or technically inadequate, use of Ki-67 and/or ProEx C IHC may be considered.
- Adherence to the recommendations below should result in the usage of p16 IHC in no more than 20% of cases in a standard laboratory, and possibly well below this, depending on local protocols.

Recommendations:

Recommendation 1: p16 IHC is recommended when the H&E morphological differential diagnosis is between precancer (high-grade SIL; –IN 2 or –IN 3) and a mimic of precancer (e.g., processes known to be not related to neoplastic risk such as immature squamous metaplasia, atrophy, reparative epithelial changes, tangential cutting).

Recommendation 2: If the pathologist is entertaining an H&E morphological interpretation of –IN 2 (under the old terminology, which is a biologically equivocal

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lesion falling between the morphological changes of HPV infection [low-grade lesion and precancer), p16 IHC is recommended to help clarify the situation. Strong and diffuse block-positive p16 results support a categorization of precancer. Negative or non-block-positive staining strongly favours an interpretation of low-grade disease or a non-HPV-associated pathology. Grading should be based on morphological features and the value of p16 is in exclusion of a high-grade lesion in the presence of a negative stain.

Recommendation 3: p16 is recommended for use as an adjudication tool for cases in which there is a professional disagreement in histological specimen interpretation, with the caveat that the differential diagnosis includes a precancerous lesion (–IN 2 or –IN 3).

Recommendation 4: The group recommends against the use of p16 IHC as a routine adjunct to histological assessment of biopsy specimens with morphological interpretations of negative, –IN 1, and –IN 3.

Recommendation 4a: SPECIAL CIRCUMSTANCE: p16 IHC is recommended as an adjunct to morphological assessment for biopsy specimens interpreted as –IN 1 or less that are at high risk for missed high-grade disease, which is defined as a prior cytological interpretation of high-grade dyskaryosis*, borderline ?high-grade*, borderline with HPV16 positive*, or borderline in glandular cells* (*these categories correspond to HSIL, ASC-H, ASC-US/HPV-16, or AGC (NOS)).

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Guidance document: p16 IHC reporting in anogenital neoplasia version 1.0, dated August 2018

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