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HPV-associated disease of the anal canal: A pathology primer

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ABSTRACT

Anal cancer is a rare disease. Screening for it in high-risk populations is based on the model borrowed from cervical cancer screening with cytology and high-resolution anoscopy-guided biopsy. Squamous cell carcinoma of the anus is associated with oncogenic HPVs, particularly type 16. Squamous intraepithelial lesions (SIL) are graded on both anal cytology and histology using 2 tiers. Low-grade SIL is reflective of a productive HPV infection; high-grade SIL is the potential precancer. High-risk HPV testing may not be an efficient screening or triage tool given the high prevalence of the virus in populations targeted for anal cancer screening. However, genotyping for HPV 16 may prove useful. On biopsy, p16 immunostaining is used to adjudicate equivocal diagnoses on routine H&E tissue sections; a positive p16 immunostain supports a diagnosis of high-grade SIL.

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Introduction

Anal cancer is rare.¹ Unlike cervical cancer, it is not an appropriate target for general population-based cancer screening. However, in specific at-risk populations, anal cancer incidence equals or exceeds the incidence of cervical cancer in US women before screening with the Pap test was initiated. Populations at highest risk for anal cancer include men who have sex with men and men and women who are HIV-infected.^{2,3} Others at higher risk include solid organ transplant recipients and women with a history of HPV-associated high-grade lower genital tract disease.⁴ These high-risk populations may be suitable for selected or targeted anal cancer screening.⁵ However, some important questions still remain unanswered regarding the effectiveness of anal cancer screening: the optimal screening approaches for anal precancers have not been identified; treatment options for anal precancer need to be improved; and treatment of anal precancer is not yet proven to reduce the incidence of progression to anal cancer.⁶

Primary anal cancer prevention is now possible with the available FDA-approved HPV vaccines.⁷ Secondary anal cancer prevention via screening is patterned after the model historically used for the cervix: screening with cytology, triage to high-resolution anoscopy (HRA) for an abnormal screen, HRA-guided biopsy, and treatment of high-grade precancers to prevent the future development of anal cancer.⁸ Cytologic screening is primarily the test for HPV-associated precancer or high-grade squamous

intraepithelial lesions (HSIL). The digital anal–rectal exam (DARE) is the screening test for anal cancer—which may be detectable via DARE as an area of pain or induration or as a mass even in its earliest stages. Biopsy diagnoses are typically considered the “gold standard” that inform patient management.

HPV is the causative agent of the majority of both cervical and anal cancers. Among the oncogenic HPVs, HPV type 16 is responsible for over 85% of anal squamous cell carcinomas.⁹ The presence of an oncogenic HPV is necessary, but not sufficient, for cancer development. The majority of humans are exposed to both oncogenic and non-oncogenic HPVs at some time in their sexual lives, usually soon after initiating sexual activity. Luckily, the majority of HPV infections are transient or remain quiescent in those with an intact immune system. The persistence of an oncogenic HPV confers the risk for development of precancer. However, despite the high prevalence of anal HPV and anal HSIL in populations at increased risk for anal cancer, progression to cancer appears relatively low.¹⁰

HPV and squamous epithelium

HPV can affect squamous epithelium in one of two ways—either as a productive infection or as a transforming infection.¹¹ In a productive infection, intact viral progeny are released as squamous cells desquamate from the surface of the epithelium. This infectious stage is usually transient and controlled by the host immune system with most active infections resolving within 1–2 years. On both cytology and histology, the morphologic appearance of a productive HPV infection is termed low-grade squamous

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intraepithelial lesion (LSIL). A transforming HPV infection occurs with persistence of an oncogenic HPV. Due to the effects of the viral oncoproteins, E6 and E7, cellular changes result that lead to genomic instability in the host cells that manifest morphologically as precancerous changes. The morphologic equivalent of a precancer is termed high-grade SIL (HSIL) on either cytology or histology. If not treated, precancers may eventually progress to cancer.

The interpretive lexicon used for cytology is the Bethesda system¹²; the LAST Project's terminology is used for histopathology.^{13–15} The Bethesda System first included anal cytology in 2001, while the LAST Project's recommendations were published in 2012. The LAST Project was a multidisciplinary, consensus effort to standardize pathology terminology and diagnostic criteria for HPV-associated squamous lesions of the lower anogenital tract. Both of these nomenclature systems recommend the term LSIL to indicate the morphologic manifestations of a productive HPV infection and HSIL to designate the morphologic manifestations of a potential precancer. Among pathology laboratories, the use of these nomenclatures has wide spread adoption for cytology and increasing uptake for histopathology. Historically, reporting of HPV-associated lesions on biopsies of squamous epithelium used the 4 tiers of mild, moderate, and severe dysplasia plus carcinoma-in-situ. This transitioned to the 3-tiered intraepithelial neoplasia (-IN) grading system (e.g., AIN1, AIN2, and AIN3) that has now evolved to the current 2-tiered system of LSIL and HSIL. This 2-tiered approach is consistent with our current understanding of the disease process.¹⁶ The intermediate category (AIN2) does not make biologic sense and is not a reproducible diagnosis among pathologists; it is composed of a mixture of low-grade and early high-grade lesions that cannot be reliably distinguished based on routine hematoxylin and eosin (H&E) morphology alone.¹¹

The microscopic features of benign and HPV-associated abnormalities of the squamous epithelium of the anal canal will be reviewed here. This will include discussion of the typical diagnoses encountered on anal cytology and histopathology reports.

Microscopic anatomy of the anal canal

The distal anal canal is lined by keratinized squamous mucosa. This region transitions to non-keratinized squamous mucosa up to the dentate line. Proximal to that is the region of the anal transformation zone (ATZ) where metaplastic squamous epithelium overlaps the colonic glandular epithelium of the distal rectum. The extent of the ATZ is highly variable. The cervix also has a similar transformation zone between squamous metaplastic epithelium and the glandular epithelium of the endocervical canal. In addition, the ATZ often displays a variant transitional epithelium

that morphologically is similar to urothelial epithelium—hence, the traditional name for the ATZ is the anal “transitional” zone.¹⁷ Benign stratified squamous epithelium can be keratinized or non-keratinized. A variety of benign, nonspecific reactive changes can be seen in the squamous epithelium. Non-keratinized squamous epithelium may show varying degrees of glycogenation, which is often depleted during reactive processes. Interstitial edema is termed spongiosis. Reactive nuclear changes include enlargement with regular nuclear contours, pale or normal chromatin, and small nucleoli. Inflammatory cells may be scattered throughout the epithelium and stroma.

For both the cervix and anus, most HPV-associated cancers arise in their transformation zones (t-zone). The leading or proximal edge of the t-zone is the squamocolumnar (SCJ) junction. In the anus, this is where the native glandular epithelium of the distal rectum meets with the squamous epithelium of the anal canal. In this region, metaplastic squamous epithelium is present (Fig. 1). Squamous metaplasia is a normal response of the tissue to irritation or trauma; however, early or immature squamous metaplasia is considered particularly vulnerable to HPV infection and oncogenic transformation. This may be due in part to the fact that the immature t-zone metaplastic epithelium is thin and the virus can easily gain access to the basal cells and basement membrane where it establishes infection. One possible reason for the difference between the rates of cervical cancer and anal cancer progression from high-grade disease is the difference in their t-zones. Unique SCJ cells have specific cell surface markers that have been identified in the cervix; similar cells have not been identified at the anal SCJ.¹⁸

Anal cytology: General considerations

Anal cytology is a good but imperfect test. Compared to the cervical Pap test, anal cytology has similar sensitivity.¹⁹ However, poor specificity and reproducibility limit its effectiveness as a screening tool.^{20,21} One of its biggest challenges is obtaining an adequate sample in the first place. Samples for anal cytology are collected “blindly” without direct visualization of the canal and the anal canal has multiple folds that can hide lesions from being readily sampled by the swab.²² Similar to the cervical Pap test, an anal cytology can be prepared as a direct or conventional smear on a glass slide then fixed or placed in a vial of liquid preservative for processing in the laboratory as a liquid-based cytology (LBC); either SurePath (BD, Franklin Lakes, NJ) or ThinPrep (Hologic, Marlborough, MA). In general, liquid-based preparations are the preferred method for anal cytology; they provide superior fixation and enhanced visualization of the cells.

Fig. 1. Anal transformation zone with reactive changes on cytology (A) and biopsy (B). The squamous metaplastic cells (arrow) have slightly enlarged nuclei with normal nuclear chromatin and contours compared with the mature squamous cells in the background. On the biopsy (B), the metaplastic squamous epithelium overlies benign rectal glandular epithelium. Normal maturation to the surface of the epithelium is present.

Fig. 2. LSIL on anal cytology (A) and biopsy (B). On both cytology and biopsy, mature squamous cells abundant cytoplasm and abnormal, dysplastic nuclei and HPV cytopathic effect are seen. (A) The majority of the cells in the field are LSIL. (B) The upper half of the epithelium shows prominent perinuclear clearing around the dysplastic nuclei, termed HPV cytopathic effect or koilocytosis.

The Bethesda system is used to report anal cytology.¹² The cytology report should include a statement regarding specimen adequacy and potentially limiting quality indicators, if present. The Bethesda system recommends a minimum of 2000–3000 nucleated squamous cells for an adequate anal cytologic sample. Pathologists do not actually count individual cells on the entire sample. Rather, the number is estimated. For example, by counting across a diameter of a ThinPrep anal cytology, 1–2 nucleated squamous cells per high-power field are needed for an adequate anal sample. Presence or absence of ATZ (defined as at least 10 rectal columnar cells and/or squamous metaplastic cells) is also included on an anal cytology report as a specimen adequacy monitor. Anal cytology that lacks an ATZ component signifies that the swab might not have gone into the anus far enough to sample cells from the t-zone where HSIL commonly occurs. Thus, a benign or negative cytology that lacks an ATZ component may be more likely to be a false-negative sample.²³

HPV-associated squamous intraepithelial lesions

Low-grade SIL is the morphologic expression of a productive HPV infection. LSIL can be caused by either low-risk or high-risk (oncogenic) HPV types. Either cytologically or histologically, mature squamous cells with abundant cytoplasm have altered or dysplastic nuclei—nuclei that show enlargement, hyperchromasia, contour, and/or chromatin abnormalities. In addition, HPV cytopathic effect or koilocytosis is frequently seen. This is a broad, well-defined perinuclear halo with an abrupt cut off to an irregular rim of peripheral cytoplasm (Figs. 2).

High-grade SIL is the morphologic manifestation of a transforming HPV infection caused by oncogenic HPVs. This is more

likely to be a non-productive infection—that is, intact virions are either not produced at all or are produced only in low copy numbers. In the t-zone, the dysplastic nuclei are typically found in immature squamous metaplastic cells. The epithelial cells are crowded, have an increased nucleus to cytoplasmic ratio (cells with large nuclei and less cytoplasm), and extend throughout the thickness of the epithelium. There is loss of the normal maturation of the cells at the surface. Mitoses can be found above their normal location in the basal layer of epithelial cells. Abnormal mitotic figures are a reflection of aneuploidy (Fig. 3).

Anal canal SCC

HPV-associated cancers of the anal canal are squamous cell carcinomas (SCC). Rectal squamous cell carcinomas are misclassified anal canal cancers that extend proximally into the rectum.²⁴ True adenocarcinomas arising in the accessory anal glands are very rare. Most adenocarcinomas found in the anal canal are rectal adenocarcinomas that have spread distally into the canal. Neither type of adenocarcinoma is associated with HPV infection.

Microscopically, SCC is characterized by malignant cells invading beneath the basement membrane into the underlying stroma. Tumor cells have increased pleomorphism and mitotic activity, including atypical forms. Nucleoli may be prominent. Since stroma is usually not present on an exfoliated cytologic sample, invasion may be inferred by the presence of increased pleomorphism (compared with HSIL), prominent nucleoli, and a tumor diathesis—the cytologic reflection of tissue destruction (Fig. 4).

Historically, the classification of anal SCC was complex and included multiple subtypes such as basaloid, cloacogenic, transitional, keratinizing, and nonkeratinizing. The latest WHO

Fig. 3. HSIL on anal cytology (A) and biopsy (B). On both cytology and biopsy, dysplastic squamous metaplastic cells with abnormal nuclei and very scant cytoplasm are present (A, arrow). On the biopsy (B), the dysplastic cells extend the full thickness of the epithelium and mitotic figures are seen above the basal layer of cells (B, arrows).

Fig. 4. Squamous cell carcinoma (SCC) on anal cytology (A) and biopsy (B). Pleomorphic, keratinized squamous cells are present on both the cytology and biopsy. On the biopsy (B), the nests of tumor cells are invading deep into the stroma; the surface epithelium is not present in the image.

classification recommends the generic term, SCC, for all squamous malignancies of the anal canal.²⁵ These are primarily HPV-driven squamous cell carcinomas; the various histologic subtypes of SCC contribute little to prognostic significance. Currently, the only two histologic subtypes of SCC that are distinct from classical SCC are a small cell (anaplastic) variant, which carries a poor prognosis and is different from neuroendocrine carcinomas, and verrucous carcinoma—a very well differentiated SCC also known as Buschke–Lowenstein tumor that is typically caused HPV 6 or 11 infection.

In the past, radical surgery with abdominal perineal resection was the primary treatment option for cancer of the anal canal. This was replaced by effective nonsurgical, sphincter-sparing combined chemoradiation therapy in the 1970s.^{26,27} The main prognostic factor of anal canal SCC is tumor stage.²⁸ T1 tumors are 2 cm or less in greatest dimension. There is no subdivision of T1 tumors of the anal canal as there is for cervical cancer. For the cervix, very early invasion (previously known as “microinvasive” SCC) can be treated with local excision such as a cold knife cone biopsy with an excellent prognosis. While local excision of early anal margin or perianal cancers is used, conservative excision of anal canal cancers is not the norm.²⁸ As more very early SCCs are detected through screening efforts using anal cytology and high-resolution anoscopy, the role of curative conservative surgical excision of anal canal tumors has once again emerged.

Anal canal superficially invasive squamous cell carcinoma (SISCCA)

Given the potential role of local surgical excision of anal canal SISCCA, the LAST Project proposed a definition of anal canal cancers with very early invasion, termed superficially invasive squamous cell carcinoma (SISCCA).^{13–15} In effect, this subdivides the T1 tumor stage. These cancers with minimal invasion are arbitrarily defined with measurements borrowed from those for cervical SISCCA: a completely excised lesion with no greater than 3 mm depth of invasion and/or 7 mm of lateral spread (Fig. 5). This is a preliminary definition to allow for the collection of reproducible data for evaluation of patient outcomes. Conservative surgical excision of these minimally invasive tumors could potentially avoid the morbidity associated with combined modality therapy.²⁹ Further study of this subset of anal cancers will determine if the subdivision of T1 lesions into SISCCA and non-SISCCA is relevant for clinical management and patient prognosis.

Equivocal findings: Squamous atypia?

Cytology and histology diagnoses are based on morphologic interpretations. Cytology assesses whole mounts of intact cells and lacks the architectural context of a 2-dimensional tissue section on

an H&E slide prepared from a biopsy. All morphologic-based interpretations are subject to interobserver variability and variation in diagnostic certainty. In addition, equivocal categories exist for both cytology and histology on routine preparations. For cytology, atypical squamous cells of uncertain significance or ASC-US is the most common equivocal category; it also is the most common abnormal cytologic interpretation. Here, the observed abnormalities fall short, either quantitatively or qualitatively, for a reproducible or confident interpretation. The differential diagnosis of ASC-US includes LSIL and reactive changes. For cervical screening, reflex HPV testing is used to adjudicate the equivocal ASC-US cytology for triage; however, this approach may not be efficient for anal cytology given the high prevalence of HPV typically seen in the populations targeted for anal cancer screening, though some have found it useful.³⁰ For atypical squamous cells that cannot exclude HSIL (ASC-H), the cytologic differential includes immature squamous metaplasia and HSIL—both have enlarged nuclei and scant amounts of cytoplasm; distinguishing between the two is based on, sometimes, subtle nuclear chromatin and irregularities in the nuclear envelop. Compared with ASC-US, there is an increased probability of finding a high-grade lesion on HRA-guided biopsy after ASC-H.³¹

On histology, equivocal diagnoses such as “atypical squamous metaplasia” may be made on routine H&E. At times, HSIL and its morphologic mimics—like atypical squamous metaplasia—cannot be reliably distinguished on H&E; the degree of diagnostic uncertainty needs to be resolved before the pathologist renders a final diagnosis. This may take the form of consultation with a colleague, reviewing additional sections through the paraffin tissue block, or supplementary stains such as immunostain.

In addition to its terminology recommendations, the LAST Project made specific proposals for biomarker use for H&E tissue

Fig. 5. On this low-power photomicrograph, a superficially invasive SCC (SISCCA) is present centrally in the anal transformation zone. It invades to a depth of less than 3 mm and is excised in the plane of section viewed here.

Fig. 6. Uses of p16 to adjudicate equivocal biopsy diagnoses. For both (A) and (B), the differential diagnosis of atypical squamous metaplasia on H&E includes high-grade SIL and a morphologic mimic of HSIL such as immature and reactive squamous metaplasia. Both (A) and (B) show nuclear crowding with decreased maturation toward the surface of the epithelium. Reactive changes including nucleoli and scattered inflammatory cells are also present. When the p16 immunostain is positive (inset A), the diagnosis of HSIL is supported. When the p16 immunostain is negative (inset B), the diagnosis of the benign reactive process of immature squamous metaplasia is supported. When the differential diagnosis on H&E is LSIL versus HSIL, p16 can be used to support a diagnosis of HSIL. On H&E, both (C) and (D) are obvious HPV-associated lesions with dysplastic nuclei and crowding; however, it is unclear if these are low-grade or an early high-grade SIL (previously referred to as AIN2). The positive p16 immunostain (inset C) supports a diagnosis of high-grade SIL—a transformed HPV infection. A negative p16 immunostain (inset D) supports a diagnosis of low-grade SIL—a productive HPV infection.

diagnoses that are equivocal high-grade precancers. In particular, the LAST project recommends p16 immunostaining in cases of diagnostic uncertainty when the differential diagnosis includes HSIL or one of its histologic mimics (Fig. 6A and B) or when a lesion is obviously HPV-associated on H&E sections but it is unclear if it is a productive low-grade lesion or an early high-grade lesion (Fig. 6C and D). p16 Immunostaining is a useful and objective tool for adjudication. It is a human tumor suppressor protein that is overexpressed secondary to the action of the viral oncoprotein, E7, which deactivates another human tumor suppressor protein, pRB. Overexpression of p16 is a surrogate marker for cellular transformation caused by the oncogenic HPVs. A positive p16 immunostain—defined as diffuse or block staining of the squamous epithelium from the basement membrane and extending at least a third of the thickness of the epithelium—supports a diagnosis of HSIL. A negative p16 immunostain is consistent with either a benign diagnosis or LSIL depending on the initial differential. Interpretation of a p16 immunostain has less interobserver variability than an H&E tissue section. Thus, improved interobserver agreement for the diagnosis of HSIL is seen with the conjunctive use of p16 immunostains with H&E morphology.³² LAST's recommendations for p16 use lead to improved accuracy and reproducibility of histologic diagnoses of precancer.^{33–35} In essence, the use of p16 immunostaining helps “polish the histologic gold standard” that informs patient management.³⁶

The distinction between LSIL (such as a condyloma or wart) and benign tissue on biopsy can also be problematic. Some pathologists may use HPV in situ hybridization (ISH) or immunostains for the L1 viral capsid proteins to assist in the diagnosis of LSIL or warts versus reactive tissue changes. Approximately, 50–60% of anal LSIL and warts will stain for these markers.³⁵ Luckily, this

differential has fewer consequences for patient management since LSIL of the anal canal is not always treated. These markers are not useful for identifying a high-grade precancer and routine use is not recommended.

Molecular markers and biomarkers: HPV testing and more

Given the very high sensitivity of high-risk HPV testing, primary HPV testing or cotesting (HPV testing along with the Pap test) has recently become the preferred cervical cancer screening method in many places worldwide. For anal cancer screening, the negative predictive value of a benign anal cytology and negative HPV test is reassuring.³⁷ Yet, due to the disproportionately high prevalence of HPV in the populations most at risk for anal cancer,³⁸ HPV testing itself may not play a prominent role in anal cancer screening or as a reflex test for triage of ASC-US, a minimally abnormal cytology. HPV testing may play more of a role in the post-HRA and post-treatment management of patients. Alternatively, HPV genotyping, particularly for HPV 16, may be helpful in the future for anal cancer screening, triage and management given its increased specificity and contribution to anal cancer.^{39,40}

Biomarkers such as p16/Ki-67 dual staining on cytologic samples also hold promise. Since cytology lacks the architectural context of a tissue section and p16 may be expressed at low levels even in benign cells, a second biomarker is needed to correctly highlight abnormal cells. Ki-67, also known as mib-1, is a proliferation marker. Since cell division and mitosis normally occur in the basal layer of squamous cells located near the basement membrane, proliferating cells are typically not found near the surface in normal squamous epithelium and thus cannot be

scraped off the surface during collection of an anal cytologic sample. The presence of both p16 expression and a proliferation marker (positive ki-67) in surface squamous cells collected for an anal cytology strongly correlates with HSIL. Since the interpretation of a cytology slide stained by both p16 and Ki-67 is more objective than routine Papanicolaou staining, it may be a beneficial adjunct, especially for the triage of ASC-US on cytology. While still a morphologic-based test, the interpretation of a dual stained cytologic sample has less interobserver variability and increased specificity than a Papanicolaou stained sample.⁴¹ It is, however, not in general use at this time.

Conclusion

Anal cancer screening is becoming more common in populations at increased risk. HPV-associated productive infections (LSIL) and potential precancers (HSIL) are diagnosed on both cytology and biopsy. The optimal methods for screening and triage still need to be verified. Recommendations from the LAST project lead to more objective and reproducible histopathologic diagnosis of equivocal findings on routine H&E. In the future, additional morphologic and molecular markers may assist with effective anal cancer screening efforts.

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