A Direct Comparative Analysis of HPV DNA with Single-Molecule RNA and p16^{INK4a} Protein Expression in Lichen Sclerosus: Implications for Diagnostics and Pathogenesis

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Introduction: Emerging evidence suggests a relationship between chronic, intermittent, occluded exposure of a susceptible epithelium to urine and male genital lichen sclerosus (MGLSc), although human papillomavirus (HPV) may also play a role. **Aims and methods:** This study investigated the association between MGLSc and HPV across the prepuce. Preputial samples from uncircumcised patients with MGLSc undergoing circumcision were tested for MGLSc distribution, HPV genotyping, RNAscope, and p16^{INK4a} detection. **Results:** Preputial samples from 9 patients with MGLSc were analyzed, with 9 distinct areas per prepuce, yielding 81 samples. These included MGLSc, non-MGLSc, and indeterminate regions. Various mucosal and beta HPV types were detected, most commonly HPV24, HPV23, HPV36, and HPV9. HPV DNA was found in all patients, and high-risk HPV types were found in 6. No significant differences were observed in total HPV ($P = .1$) or oncogenic HPV ($P = .6$) between MGLSc and non-MGLSc tissues. Transcriptionally active HPV was absent in all samples on the basis of independent RNAscope and p16^{INK4a} staining. **Discussion:** HPV DNA was detected in a mosaic pattern across the prepuce, with no significant differences between MGLSc and non-MGLSc skin. The absence of transcriptional activity suggests that HPV in MGLSc is incidental and may not contribute toward pathogenesis.

Keywords: Gene transcription, Inflammatory skin diseases, Papillomavirus, RNA biology, Transcription

INTRODUCTION

Male genital lichen sclerosus (MGLSc), an acquired skin disorder with major clinical consequences, poses a significant risk factor for penile intraepithelial neoplasia (PeIN) and penile squamous cell carcinoma (PeSCC) (Kravvas et al, 2022a, 2018). Although the precise etiopathogenesis of MGLSc remains contested, emerging evidence suggests a relationship with chronic, intermittent, occluded exposure of a susceptible epithelium to urine (Al-Niaimi and Lyon, 2013; Chan et al, 2022; Doiron and Bunker, 2017; Kravvas et al, 2022a; Panou et al, 2022).

The relationship between human papillomavirus (HPV) and MGLSc has been a topic of considerable investigation, largely owing to the overlapping presence of both conditions in PeIN and PeSCC (Kravvas et al, 2022a, 2018; Shim et al, 2021). Although HPV infection is a well-established driver of genital neoplasia, its role in MGLSc remains uncertain (Bunker, 2019; Kravvas et al, 2022b, 2018).

However, the variable HPV findings between previous studies and the high proportions of HPV-negative MGLSc cases have led several investigators, including us, to propose the notion that HPV may not have a pathogenic role in MGLSc but is rather present as a passenger (Aidé et al, 2010; D'Hauwers et al, 2012; Edmonds et al, 2011; Shim et al, 2020).

In addition to these variable results, there are also fundamental questions regarding the presence of HPV DNA in tissue samples and transcriptionally active HPV that may correlate with the etiology of disease. Until recently, detecting transcriptionally active viral DNA was a technical challenge. The advent of single-molecule RNAscope technology has enabled in situ detection of transcriptionally active RNA

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Abbreviations: HPV, human papillomavirus; HR, high-risk; MGLSc, male genital lichen sclerosus; PeIN, penile intraepithelial neoplasia; PeSCC, penile squamous cell carcinoma

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for viruses such as HPV. Kravvas et al (2025) recently examined the expression of transcriptionally active RNA in a variety of male urogenital disorders.

This investigation had 2 key aims: (i) to assess the prevalence and distribution of HPV DNA in MGLSc versus non-MGLSc penile skin and (ii) to examine its potential role in MGLSc pathogenesis by comparing HPV DNA detection through sequencing with the outcomes of RNAscope and $p16^{INK4a}$ expression, in situ, in the same tissues. The outcome of such a comparison could have important clinical implications because the detection of HPV DNA may influence patient management.

Our findings demonstrated no difference in the tropism of HPV DNA between MGLSc and non-MGLSc tissues and showcased the presence of DNA from multiple HPV types, arranged in a mosaic pattern. Despite the presence of HPV DNA in all 9 patients, no evidence of transcriptionally active HPV was detected in these tissues using RNAscope and $p16^{INK4a}$ staining.

RESULTS

All 9 patients included in the study were uncircumcised, with ages ranging from 23 to 88 years (median = 40 years, mean = 48 years) (Table 1). Assessment of the H&E-stained slides adjacent to all the sections showed no evidence of a productive HPV infection by bright-field microscopy, with no findings of microlesions, koilocytosis, dysplastic cells, or other features indicative of HPV infection. In addition, all the slides were also negative for $p16^{INK4a}$, with positive and negative controls validating the accuracy of the analysis.

DNA sequencing and genotyping showed that among the 9 patients, a variety of mucosal and beta HPV types were detected, with HPV24, HPV23, HPV36, and HPV9 being the most frequently encountered types. Each patient harbored between 2 and 7 HPV types, with individual sections showing anywhere from no HPV to up to 7 types per 0.79 mm^2 area. All 9 patients harbored additional, unidentifiable HPV types.

A total of 4 oncogenic or probably oncogenic types (classes 1 and 2, high-risk [HR]) were observed in 6 patients (HPV18, 31, 34, 51, 52, 66, and 68/73), whereas 3 patients did not harbor any HR HPV types. Four patients harbored a single type of HR HPV, and 2 patients harbored 2 types of HR HPV. The presence of HPV DNA was identified in 80% of MGLSc samples, in 94% of non-MGLSc samples, and in 80% of indeterminate areas. Overall, 82% of all samples harbored at least 1 HPV type. The difference in HPV between MGLSc and non-MGLSc areas was not found to be statistically significant ($P = .1$).

The presence of HR HPV was observed in 16% of MGLSc samples, in 12% of non-MGLSc samples, and in 10% of indeterminate areas. Overall, 15% of the samples harbored at least 1 oncogenic HPV type. The difference in HR HPV between MGLSc and non-MGLSc areas was found to be not statistically significant ($P = .6$). Summaries of these findings are provided in Table 1 and illustrated in Figure 1.

Table 1. Patient Demographics and Tissue Analysis for MGLSc and HPV

Patient	Age, y	Percentage of Tissue with MGLSc (%)	Percentage of Areas with HPV DNA (%)	Percentage of Areas with HR HPV DNA (%)
1	59	70	77.8	66.7
2	38	80	100	22.2
3	71	70	88.9	11.1
4	23	70	100	11.1
5	62	20	100	0
6	54	80	77.8	0
7	88	100	66.7	0
8	30	30	66.7	11.1
9	25	90	55.6	11.1

Abbreviations: HPV, human papillomavirus; HR, high-risk; MGLSc, male genital lichen sclerosus.

This table displays data for each of the 9 patients in the study, including patient age, the percentage of tissue with MGLSc, the percentage of tissue testing positive for HPV DNA, and the percentage of tissue testing positive for HR HPV types.

Comparative analysis of HPV DNA sequencing, RNAscope, and $p16^{INK4a}$ staining

RNAscope allows the detection of transcriptionally active RNA, whereas $p16^{INK4a}$ serves as a surrogate marker for biologically active HR HPV infection (da Mata et al, 2021; Wang et al, 2012).

We compared the presence of HPV DNA with $p16^{INK4a}$ expression and RNAscope results for both low-risk and HR HPV. Although DNA sequencing revealed the presence of multiple HPV strains in all 9 patients, including HR HPV in 6 of them, RNAscope and $p16^{INK4a}$ analysis did not detect any evidence of transcriptionally active HPV in any of the 9 patients (Figure 2). This finding highlights a crucial distinction between the mere presence of viral DNA and the detection of biologically active virus, with significant implications for understanding disease pathogenesis. Combined results from the comprehensive detection of HPV in all 9 patients and across all the applied techniques are illustrated in Figure 3.

DISCUSSION

In a previous study, Kravvas et al (2025) demonstrated the expression of transcriptionally active HR and low-risk HPV using RNAscope and correlated it with $p16^{INK4a}$ protein expression. In this study, we complemented that work with DNA sequencing, another more commonly used technique for detecting HPV DNA, in a smaller cohort of samples. We further suggest that measurement of transcriptionally active HPV RNA in HPV-related diseases should be set as a gold standard.

Methods for HPV detection

A variety of techniques have been used for detecting HPV, with the most common being the PCR (Nasca et al, 2006; Sarier et al, 2024, 2023; von Krogh et al, 2002; Zhang et al, 2016). Variants of PCR, such as broad-spectrum and type-specific assays, also allow for the identification of multiple HPV types within a single sample (Carvalho et al, 2010; Li et al, 2023). Serological assays that detect antibodies against HPV proteins are also used in epidemiological studies to assess past exposure but are not suitable for diagnosing

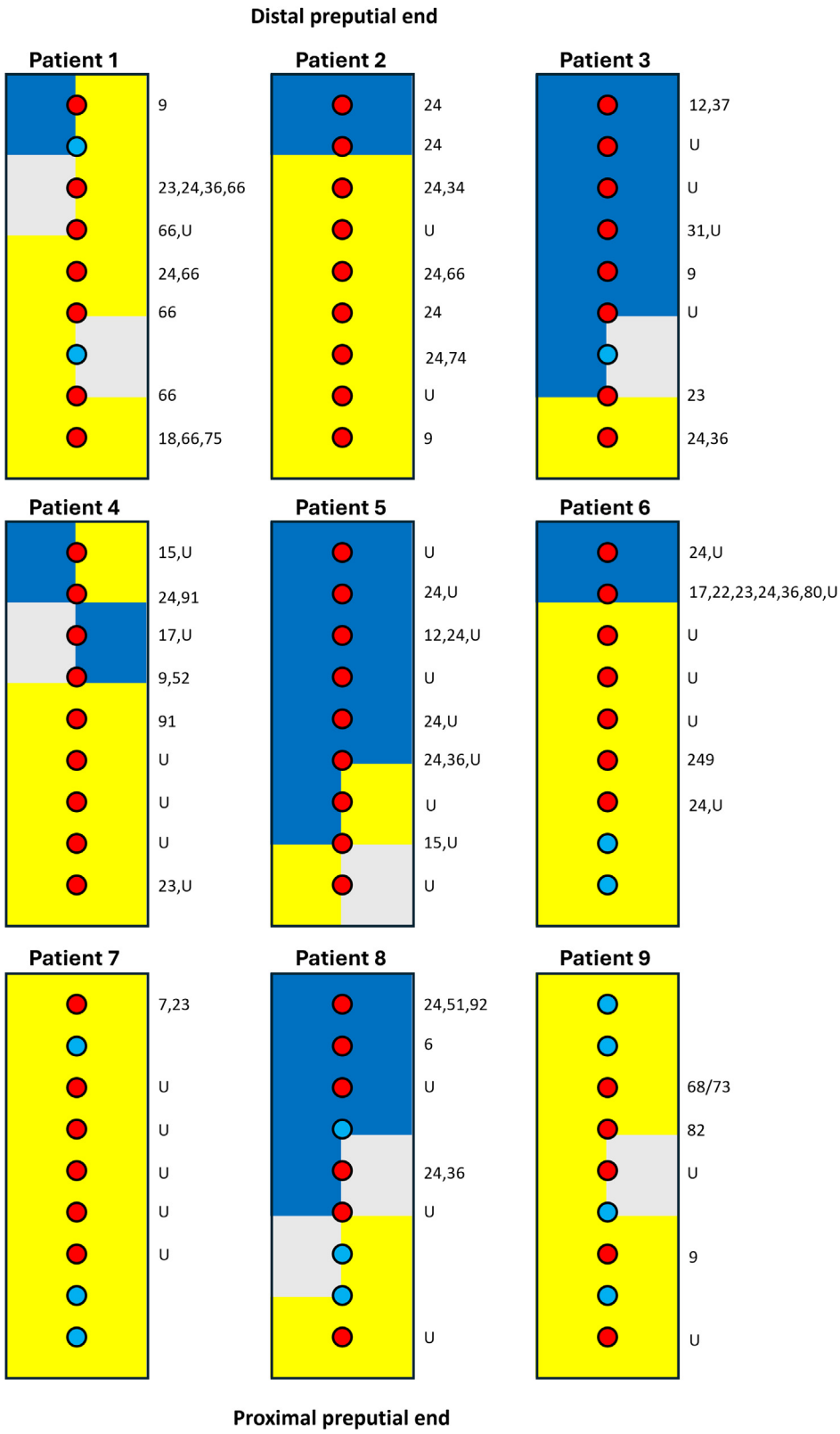


Figure 1. The distribution of HPV in preputial samples from 9 patients with MGLSc. Each rectangle represents a preputial section from 1 of the 9 study patients. Red circles indicate HPV-positive sites, whereas cyan circles represent HPV-negative sites. The numerals next to each rectangle specify the HPV type detected at each site; the letter "U" denotes unidentified HPV types. Regions of MGLSc are shaded in yellow, non-MGLSc skin is presented in blue, and indeterminate areas are in white. HPV, human papillomavirus; MGLSc, male genital lichen sclerosus.

active infections (Mesher et al, 2016; Silverberg et al, 2006). In situ hybridization is another method that can detect HPV DNA directly in tissue samples, preserving tissue morphology and allowing for visualization of viral localization, although it is less sensitive than PCR (Kravvas et al, 2025; Rooper et al, 2016; Siti-Aishah et al, 2000). Immunohistochemistry with $p16^{INK4a}$ serves as an indirect marker for HR HPV; using a direct approach, Kravvas et al (2025) recently showed that

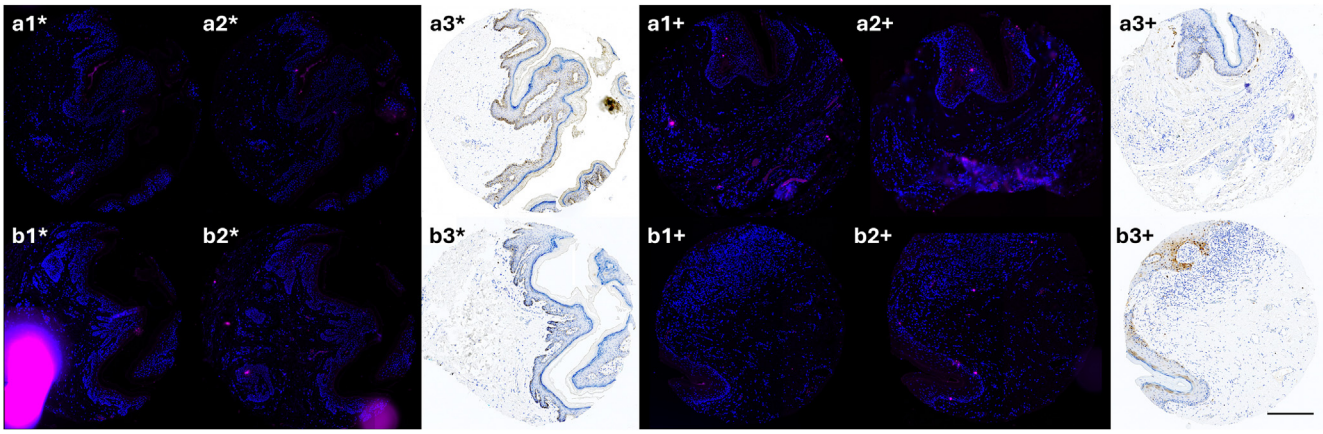
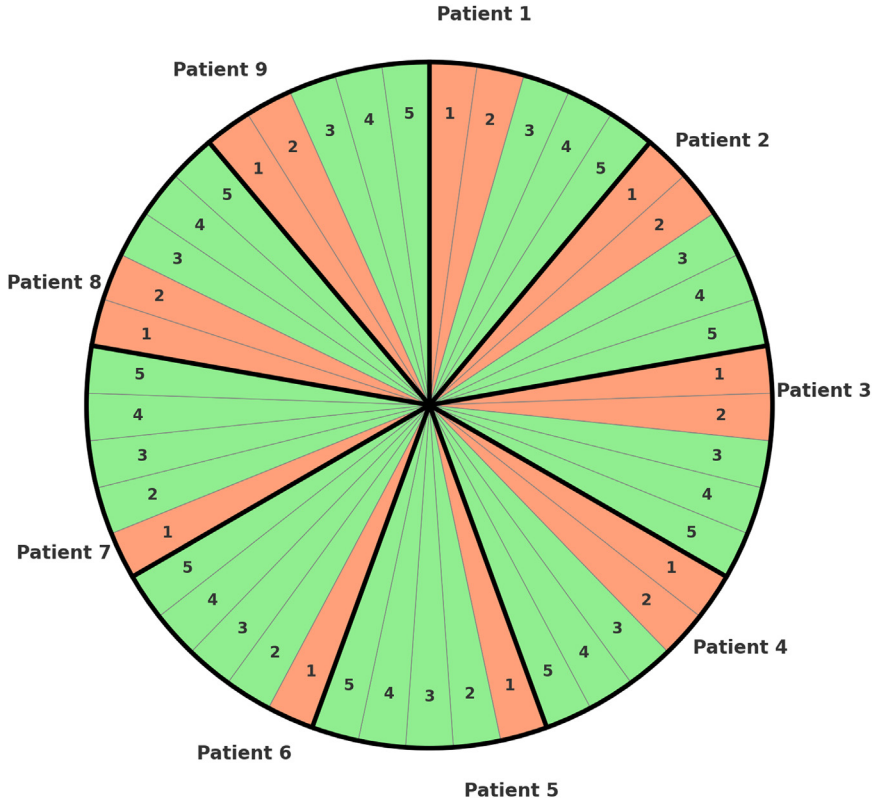


Figure 2. RNAscope and p16^{INK4a} staining in MGLSc and non-MGLSc penile tissues. Representative images of sister sections from 4 tissue cores obtained from MGLSc and non-MGLSc penile skin from 2 patients. Sister sections were stained for HR and LR HPV RNAscope and for p16^{INK4a}. All the sections demonstrate negative or weak and patchy staining, indicating the absence of active HPV infection. This finding is observed despite DNA sequencing revealing the presence of multiple HR and LR HPV types in the corresponding tissues. Letters represent individual patients, numerals represent the technique of staining utilized (1 = RNAscope for LR HPV, 2 = RNAscope for HR HPV, and 3 = p16^{INK4a}), and symbols indicate the type of tissue studied (* = non-MGLSc skin, + = MGLSc skin). These figures are modified versions from Kravvas et al (2024). Bar = 600 μm (this bar applies to all the histologic images presented in this figure). HPV, human papillomavirus; HR, high-risk; LR, low-risk; MGLSc, male genital lichen sclerosus.

p16^{INK4a} is a good surrogate for HPV infection. More recently, RNA in situ hybridization with RNAscope, a highly specific and sensitive method that detects the presence of transcriptionally active RNA from active infections, has been employed (Kravvas et al, 2025). Next-generation sequencing provides a high-throughput approach for detecting a broad array of HPV types and variants, offering a comprehensive view of HPV diversity within a sample, although it is more costly and complex than traditional methods (Andersen et al, 2022; Mühr et al, 2021; Wagner et al, 2019). Each of these techniques has benefits and limitations, and the choice of method depends on the clinical or research question, the need for sensitivity and specificity, and the available resources.

In this study, we combined the modern, sensitive, and specific techniques of DNA sequencing, single-molecule

Figure 3. Comprehensive detection of HPV in all 9 patients across all the applied techniques. This pie chart is divided into 9 main segments, each representing 1 patient. Each main segment is further divided into 5 subsegments corresponding to the following subheadings: (i) LR HPV DNA, (ii) HR HPV DNA, (iii) RNAscope for LR HPV, (iv) RNAscope for HR HPV, and (v) p16^{INK4a}. The color of each subsegment indicates the detection status of HPV using the respective technique. Light green denotes that no HPV was detected, whereas orange indicates the detection of HPV. Segments are delineated by bold lines, whereas subsegments are separated by finer, thinner lines. Note: Our data were processed using artificial intelligence software (ChatGPT, version 4.0) to generate this figure. HPV, human papillomavirus; HR, high-risk; LR, low-risk.



RNAscope, and $p16^{INK4a}$ with sequential patient samples to covalidate the presence of transcriptionally active or inactive HPV in MGLSc and non-MGLSc tissues.

Transcriptionally active versus dormant viral detection in patient tissue samples

The transcriptional activity or inactivity of HPV could be important for the etiology of HPV-related diseases (Adelstein et al, 2009). For example, it has been shown for oropharyngeal squamous cell carcinoma that transcriptionally active HPV is correlated with prognosis (Larque et al, 2014). DNA sequencing and PCR are commonly used methods of detecting HPV DNA in clinical samples; however, DNA detection does not necessarily imply transcriptionally active virus (Śnietura et al, 2010). Other studies have suggested an association between HPV and MGLSc, particularly through shared links with PeIN and PeSCC; however, these have also either used protein expression for $p16^{INK4a}$, a surrogate for HPV infection, or PCR-based techniques (Drut et al, 1998; Prowse et al, 2008). Our findings propose an alternative perspective, suggesting that covalidation using multiple techniques for HPV detection in clinical samples can provide greater insight into the causal relationship between infection and disease.

Active, latent, and "cleared" infections

HPV infections in genital skin are thought to exist in a few states: active, latent, and cleared. An active infection is characterized by viral replication and often by histological changes, such as koilocytosis, and may present clinically as warty lesions (Kravvas et al, 2025). In contrast, latent infections are defined by the presence of HPV DNA within basal epithelial cells without active viral replication or associated histopathological changes. These infections may persist undetected and can be reactivated under certain conditions (von Krogh et al, 2002). Finally, the concept of "cleared" infection is somewhat misleading. Evidence suggests that HPV DNA may remain in basal cells indefinitely, albeit at undetectable levels, with the potential to re-emerge. Therefore, what is often termed "clearance" may, in fact, represent suppression and latency rather than complete eradication of the virus (Doorbar, 2013; Gravitt, 2012; McBride, 2024; Roehr, 2012). This continuum of infection states provides critical insight into the role or lack thereof of HPV in MGLSc. Our findings indicate a high prevalence of HPV DNA in both MGLSc and non-MGLSc tissue without evidence of active infection, underscoring the incidental nature of HPV presence in this context.

HPV prevalence in MGLSc and non-MGLSc tissues

Our results demonstrate a high overall prevalence of HPV in both MGLSc (80%) and non-MGLSc (94%) preputial tissues. The absence of a statistically significant difference in HPV presence between MGLSc and non-MGLSc regions ($P = .1$) suggests that HPV infection is unlikely to play a direct role in MGLSc pathogenesis. Moreover, HR HPV types were detected in a minority of samples (16% in MGLSc and 12% in non-MGLSc areas), with no significant difference in distribution ($P = .6$). These findings align with those of previous studies indicating that HPV detection in MGLSc lesions likely represents incidental colonization rather than a causal factor in disease development (Kravvas et al, 2025; Shim et al, 2020).

In addition, the detection of multiple HPV genotypes in all patients, regardless of MGLSc involvement, highlights a mosaic pattern of HPV distribution within penile tissues. This widespread presence of diverse HPV types, including HPV4, 23, 36, and 9—none of which are typically associated with oncogenesis—further supports the notion that HPV presence in MGLSc lesions is an incidental epiphenomenon rather than a pathogenic driver. This conclusion is reinforced by the absence of markers of productive HPV infection, such as koilocytosis or dyskeratotic cells, on histology as well as the lack of $p16^{INK4a}$ positivity in MGLSc tissues. Together, these findings indicate an absence of active HR HPV–driven cellular changes within MGLSc lesions.

Oncogenic HPV and MGLSc

Although a subset of samples harbored oncogenic HPV types (HPV18, 31, 51, and 68), these findings were scattered across patients and showed no preferential distribution in MGLSc areas. Notably, only 11.1% of total samples contained HR HPV DNA, and most patients either harbored single oncogenic HPV types or none at all. This sporadic presence of oncogenic HPV further implies a limited role for HPV in the pathogenesis of MGLSc. Although HPV has been implicated in PeIN and PeSCC, the relatively low incidence of HR HPV in MGLSc samples suggests that HPV infection alone may not significantly contribute to malignant transformation within MGLSc lesions (Kravvas et al, 2025, Kravvas et al, 2022a; Shim et al, 2020).

Implications, limitations, and future directions

Our findings challenge the proposed etiological connection between HPV and MGLSc, suggesting instead that HPV presence in MGLSc tissues may reflect incidental colonization of a compromised epithelial barrier rather than an active role in MGLSc pathogenesis. This conclusion stands in contrast to HPV-driven conditions, where a strong association with HR HPV is consistently linked to specific histopathological alterations.

A key limitation of this study is the small sample size, which restricts the generalizability of our findings. Future research involving larger, well-matched cohorts and controlled comparisons between MGLSc and non-MGLSc populations is essential to validate these findings and explore any potential indirect interactions between HPV and MGLSc that might influence disease development. In addition, examining molecular markers and inflammatory pathways beyond HPV could yield critical insights into the pathogenesis of MGLSc and its progression to PeIN or PeSCC. Such approaches may reveal underlying mechanisms unrelated to HPV, advancing our understanding of MGLSc and opening new avenues for targeted therapeutic interventions.

HPV of multiple genotypes, including HR types, was detected at a high frequency through DNA in both MGLSc and non-MGLSc penile skin. Although HPV DNA was consistently identified in all patients, transcriptionally active HPV was completely absent in both MGLSc and non-MGLSc tissues, as confirmed by RNAscope and $p16^{INK4a}$ analysis.

These findings argue for a shift in focus toward (i) covalidation of existing methods of HPV detection and (ii) alternative and more compelling pathogenic mechanisms in the etiology and, importantly, management of MGLSc.

MATERIALS AND METHODS

This prospective study was undertaken at a specialist multidisciplinary (andrology, dermatology, and histopathology) center as part of a program focussed on the pathogenesis of MGLSc, PeIN, and PeSCC.

This study was performed in accordance with the Declaration of Helsinki. This human study was approved by Joint Research Office (approval 20/SC/0037). All adult participants provided written informed consent to participate in this study.

Nine uncircumcised male patients with a clinical diagnosis of MGLSc who were scheduled for circumcision as part of routine medical care were enrolled in the study.

Fresh-frozen preputial samples were collected at the time of circumcision from 9 patients with clinically diagnosed MGLSc. The samples were divided into grids, and biopsies were obtained to determine the spatial distribution of MGLSc, as previously described (Kravvas et al, 2022a). In addition, punch biopsies of 1 mm in diameter were obtained from preterminal areas in each prepuce for HPV genotyping, in situ hybridization with RNAscope, and p16 ^{INK4a} staining.

The 9 preputial tissues were sampled in 9 areas each, making a total of 81 samples that were used for DNA sequencing and genotyping. Of these 81 samples, 44 were from MGLSc skin, 17 were from non-MGLSc skin, and 20 were from indeterminate areas. None of the 9 patients were noted to have viral warts upon clinical examination, and none had received vaccination against HPV.

HPV-DNA detection and genotyping were performed exclusively on tissue samples obtained from preputial samples. HPV DNA amplification, detection, and genotyping were conducted using the following methods. A total of 65 HPV genotypes were evaluated, comprising 25 beta HPV types, 25 genital HPV types, and 15 mucosal HPV types. For beta papillomaviruses, RHA Kit Skin (beta) HPV, which utilizes broad-spectrum PCR and reverse hybridization with line probe technology for genotyping 25 known beta papillomavirus types (ie, HPV5, 8, 9, 12, 14, 15, 17, 19-25, 36−38, 47, 49, 75, 76, 80, 92, 93, and 96) (de Koning et al, 2006), was performed. For genital HPV types, RHA Kit HPV SPF10-LiPA25, version 1, which includes SPF10-PCR, SPF10-DEIA assay for detection of PCR-amplified DNA of over 67 HPV types, and the HPV-LiPA assay for the simultaneous identification of 25 HPV genotypes (ie, HPV6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, 74) (Geraets et al, 2014), was performed. In addition, amplimers from samples positive with the DEIA and negative on the LiPA were tested using an additional strip containing probes for another 15 mucosal HPV types (ie, HPV26, 30, 61, 62, 67, 69, 71, 82, 83, 84, 85, 87, 89, 90, and 91).

The HPV genotypes were classified on the basis of the International Agency for Research on Cancer classification system. HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 were categorized as carcinogenic (class 1). HPV34, 53, 66, 68/73, and 70 were classified as probably or possibly carcinogenic (class 2A/B). Meanwhile, HPV6, 11, 40, 42, 43, 44, 54, and 74 were designated as noncarcinogenic (class 3).

All adjacent sections were subjected to further staining with H&E and p16^{INK4a} immunohistochemistry, the latter serving as a surrogate marker of HR HPV infection (Sritippho et al, 2016).

In situ hybridization with RNAscope

Formalin-fixed and paraffin-embedded penile tissue samples from the same preputial samples were also utilized for the detection of HR and low-risk HPV using RNAscope.

The selected probes targeted the 16 most prevalent HR HPV types (HPV-HR18, 312598, Advanced Cell Diagnostics) and the 10 most common low-risk HPV types (HPV-LR10, 314558, Advanced Cell Diagnostics). *UBC*, a highly transcribed and widely expressed gene in human tissues, served as the positive control (Hs-UBC, 312028, Advanced Cell Diagnostics), whereas *dapB*, a bacterial gene not expressed in human tissues, was used as the negative control (dapB, 312038, Advanced Cell Diagnostics) (Bianchi et al, 2019; Pavelka et al, 1997).

Comprehensive details regarding the method of tissue array construction and the RNAscope technique are available in the literature (Kravvas et al, 2025).

STATISTICAL ANALYSIS

Descriptive statistics, including means, medians, and percentages, were used to summarize patient demographics, tissue characteristics, and HPV detection rates.

The proportions of samples testing positive for HPV were compared using chi-square tests. A significance threshold of $P < .05$ was used to determine statistical significance. All statistical tests were 2 tailed.

ETHICS STATEMENT

This study was performed in accordance with the Declaration of Helsinki. Collection of human tissue samples for this study was approved as part of the study protocol. This human study was approved by University College London Hospitals/University College London Joint Research Office (approval 20/SC/0037). All adult participants provided written informed consent to participate in this study.

DATA AVAILABILITY STATEMENT

Data are available within the article.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: GK, MdK, SJG, AM, ML, AA, CBB; Data Curation: GK, ML, MM, AA, CBB; Formal Analysis: GK, AA, CBB; Funding Acquisition: GK, CBB; Investigation: GK, BX, CG, HvdM, EvdM, MdK, AF, AH, ML, MM, AA,

CBB; Methodology: GK, BX, AA, CBB; Project Administration: GK, CBB; Resources: GK, HvdM, HA, AM, ML, MM, AA, CBB; Software: GK, BX, AA, CBB; Supervision: AA, CBB; Validation: GK, BX, AA, CBB; Visualization: GK, BX, AA, CBB; Writing − Original Draft Preparation: GK, AA, CBB; Writing − Review and Editing: GK, BX, CG, HvdM, EvdM, MdK, SJG, AF, AH, HA, AM, ML, MM, AA, CBB

DECLARATION OF GENERATIVE ARTIFICIAL INTELLIGENCE (AI) OR LARGE LANGUAGE MODELS (LLMs)

During the preparation of this work, the authors used OpenAI ChatGPT (version January 2024) to create Figure 3. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

ChatGPT was asked to generate a pie chart using the following prompts: Make a pie chart divided into 9 main segments, each representing 1 patient. Each main segment should be subdivided into 5 subsegments, each corresponding to the following subheadings (each representing a human papillomavirus [HPV] identification technique): 1 = low-risk (LR) HPV DNA, 2 = high-risk (HR) HPV DNA, 3 = RNAscope for LR HPV, 4 = RNAscope for HR HPV, and 5 = $p16^{INK4a}$ (name them 1−5). The color of each subsegment should indicate the detection status of HPV using the respective technique. Light green denotes that no HPV was detected, whereas light orange indicates the detection of HPV. Segments are separated by bold lines, whereas subsegments are separated by finer, thinner lines. Patient 1: only LR HPV DNA and HR HPV DNA were positive. Patient 2: only LR HPV DNA and HR HPV DNA were positive. Patient 3: only LR HPV DNA and HR HPV DNA were positive. Patient 4: only LR HPV DNA and HR HPV DNA were positive. Patient 5: only LR HPV DNA was positive. Patient 6: only LR HPV DNA was positive. Patient 7: only LR HPV DNA was positive. Patient 8: only LR HPV DNA and HR HPV DNA were positive. Patient 9: only LR HPV DNA and HR HPV DNA were positive. Add labels: patients 1−9, for each segment, and labels 1−5 for each subsegment. Place the subsegment labels near the outer edge of the pie chart but within the pie chart, near the middle of each arc. Arrange the subsegments in a clockwise manner in the following order: 1 = LR HPV DNA, 2 = HR HPV DNA, 3 = RNAscope for LR HPV, 4 = RNAscope for HR HPV, and 5 = $p16^{INK4a}$. Make the labels of the subsegments in bold. keep the subsegment divisions in regular print. Make the outer edge of the circle in bold. Make the subsegment dividing lines in lighter gray and finer width.

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