Original Article doi: 10.5146/tjpath.2026.14524

HPV Detection by RT-PCR in Laryngeal Squamous Cell Carcinoma: Correlation with p16 Expression, Histopathology, and Prognosis

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Turk Patoloji Derg 2026, 42:I-VIII **Received:** 03.08.2025 **Accepted:** 28.07.2026 **Published Online:** 02.10.2026

ABSTRACT

Objective: The aim of the study was to determine HPV incidence among laryngeal squamous cell carcinomas (LSCC) using HPV E6/E7 mRNA RT-PCR and to compare diagnostic capabilities of p16 immunohistochemistry against it. With the data gathered, the relationship between HPV status, pathological and clinical parameters will be assessed.

Material and Methods: This retrospective, single-centred study included 112 laryngectomy cases tested for HPV E6/E7 mRNA using RT-PCR, which detects transcripts of 14 high-risk HPV types, and additionally identifies the presence of HPV 16 and 18/45-specific transcripts. p16 protein immunohistochemistry was also performed, and overall results were compared with clinical and pathological data.

Results: p16 immunoreactivity was observed in 18 cases (16%). In one case, more than 70% of the tumour cells exhibited strong staining. The p16 immunohistochemistry (IHC) score was above 6/9 in 5 cases (28% of p16-positive cases, 4% of all cases). Despite p16 reactivity, none of the cases demonstrated transcriptionally active HPV with RT-PCR tests. The RT-PCR analysis result was positive for HPV type 16 in one case, with no p16 immunoreactivity observed in the tumour cells.

Conclusion: In our study, the findings suggest that HPV has a minimal effect on LSCC carcinogenesis in Türkiye. Our findings suggest that p16 immunohistochemistry may not be a suitable surrogate marker for HPV detection in LSCC. Additionally, p16 IHC positivity was associated with decreased survival. This suggests that p16 expression could potentially be used as a prognostic marker, although its clinical significance requires fur-ther validation.

Keywords: Squamous cell carcinoma, Head and neck cancer, Laryngeal cancer, Human papillomavirus (HPV), RT-PCR, p16

INTRODUCTION

Laryngeal squamous cell carcinoma (LSCC) accounts for 90% of primary laryngeal malignancies and about 2% of cancers worldwide. In Turkey, it is the sixth most common cancer in men, representing 3% of all malignancies (1,2). While smoking is the primary risk factor, the role of human papillomavirus (HPV) in LSCC remains unclear. Reported HPV prevalence in LSCC varies from 3% to 60%, influenced by regional differences and detection methods (3,4). The biological plausibility of HPV involvement in LSCC is supported by the well-established causative role of high-risk HPV types in oropharyngeal squamous cell carcinoma (OPSCC) and by the occurrence of recurrent respiratory papillomatosis — caused by low-risk HPV types 6 and 11 — in laryngeal tissue. However, because high-risk HPV types can be detected in morphologically normal laryngeal mucosa, the detection of HPV DNA alone is insufficient to confirm an aetiologically active infection (5,6). Transcriptionally active infection, evidenced by E6/E7 oncogene mRNA expression, is required to implicate HPV in carcinogenesis.

HPV detection methods include Polymerase Chain Reaction (PCR) for viral DNA and Reverse Transcription PCR (RT-PCR) or in situ hybridisation (ISH) for E6/E7 mRNA, which signal active viral oncogenesis. E6/E7 mRNA tests are considered the gold standard due to their higher specificity (4). Immunohistochemistry (IHC) for $p16^{INK4a}$ (p16) protein is a cost-effective and widely used surrogate marker for HPV-driven carcinogenesis in oropharyngeal cancers and cancers of unknown primary. However, p16 overexpression can occur via HPV-independent mechanisms, introducing the risk of false positives when applied

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outside the oropharynx (7-10). This study aims to assess HPV prevalence in LSCC using RT-PCR for E6/E7 mRNA and to evaluate the diagnostic performance of p16 IHC as a surrogate marker, correlating the findings with clinical and pathological data.

MATERIAL and METHODS

Case Selection and Patient Demographics

A total of 175 laryngectomy specimens received and reported at the Department of Medical Pathology of our tertiary centre university hospital were initially included in the study. Cases with tumour centred in locations outside the larynx, i.e. hypopharyngeal tumours with the tumour centre in the piriform sinus, were excluded. Among the remaining cases, those without sufficient tumour tissue (less than 4 mm^2) and tumours with signs of extensive autolysis or decalcification were excluded from the study. After applying the inclusion and exclusion criteria, 112 cases were included in the final analysis.

Patient data, including age, gender, histological grade, tumour location, size, pathological stage, lympho-vascular and perineural invasion, and surgical margin information, were obtained from pathology reports. Information on smoking and alcohol use, as well as chemoradiotherapy history, was obtained from the MUH Ear, Nose, and Throat (ENT) Department's patient files. Survival data were collected from the Turkish Ministry of Health's death notification system. The study, with application number 09.2019.140, was approved by the university clinical research ethics board.

HPV RT-PCR

In our study, the Aptima HPV Assay, approved by the FDA for cervical cancer screening and manufactured by Hologic Gen-Probe, Inc., was used to detect Human Papillomavirus (HPV) in formalin-fixed paraffin-embedded (FFPE) tissue. This method can identify E6/E7 mRNA transcripts of 14 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) through RT-PCR. Additionally, the system detects HPV 16 and 18/45-specific transcripts, though it cannot differentiate between HPV 18 and 45.

Results were classified as negative, positive, or invalid based on the signal-to-cutoff (S/CO) ratio of HPV RNA:DNA hybrids compared to internal control RNA:DNA hybrids. The internal control threshold value was derived from the average Relative Light Units (RLU) emitted by a negative calibrator.

Chromogenic In Situ Hybridisation (CISH)

The ZytoFast® HPV-CISH System by ZytoVision GmbH was used for detecting HPV DNA in FFPE tissue. The tissue microarray technique was employed to evaluate more cases. The tissue was then extracted using the Tissue-Tek Quick-Ray device and transferred to recipient blocks. The same blocks were used for p16 immunohistochemical tests. As an external control, blocks of cervical squamous cell carcinoma and high-grade squamous intraepithelial lesions of patients previously confirmed to have HPV 16 infection on cervical smears using the Aptima HPV Assay were used for validation. CISH slides were examined under a light microscope for the presence of brown DAB precipitate in the nucleus, indicating HPV DNA.

Immunohistochemistry for p16

Staining was performed using the fully automated Ventana Benchmark Ultra system by Ventana Medical Systems. For p16 immunohistochemistry, Roche Diagnostics' CINtec® Histology, mouse monoclonal anti-p16 antibody clone E6H4, was utilised. Stained slides were evaluated under a light microscope. Cytoplasmic and/or nuclear brown chromogen accumulation was considered significant. Tumour sections were assessed based on staining distribution, intensity, and pattern. Two criteria were used to determine p16 positivity separately.

Firstly, nuclear and cytoplasmic moderate to strong staining in more than 70% of tumour cells was considered positive. In the second method, the p16 IHC score was calculated based on staining percentage and intensity (Figure 1) The p16 IHC score was calculated by multiplying these two values. For the staining proportion score, a value of 1 was assigned when the staining proportion was between 0% and 35%, 2 for proportions between 36% and 70%, and 3 for proportions between 71% and 100%. For the staining intensity score, a value of 1 was given for weak staining, 2 for moderate staining, and 3 for strong staining. The final p16 IHC score was determined by multiplying the proportion score with the intensity score. The score was considered positive if it was 6 or above out of 9.

Statistical Analysis

IBM® SPSS® Statistics version 24.0 was used for statistical analysis. A p-value of <0.05 was considered statistically significant in all analyses. Survival analysis was performed using the Kaplan-Meier method, and differences between groups were assessed using the log-rank test. Associations between p16 IHC positivity/score and clinicopathological parameters were assessed using Fisher's exact test and the Mann-Whitney U test.

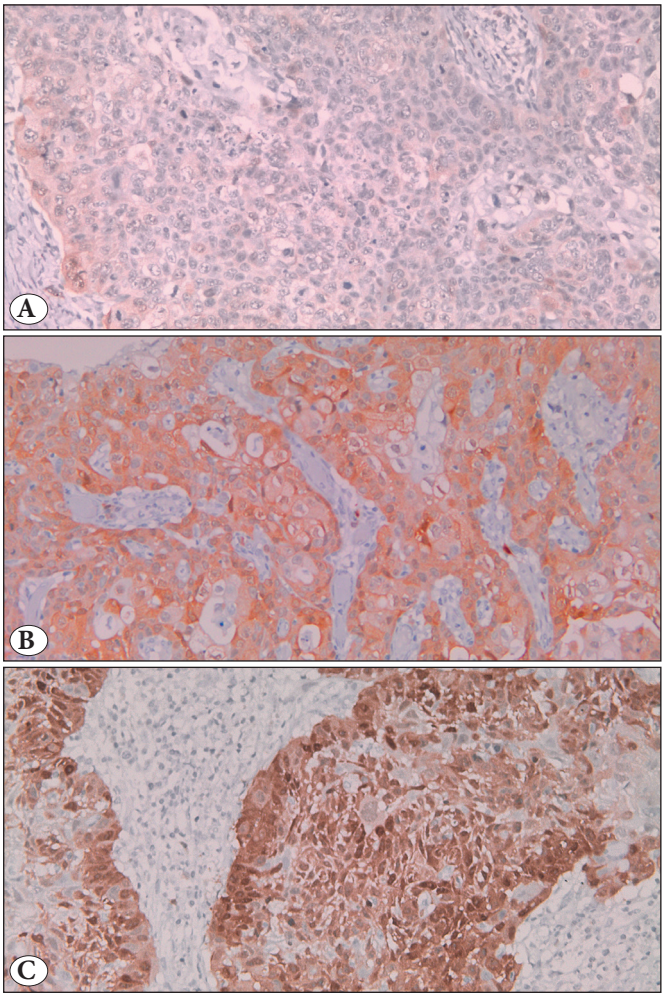


Figure 1: Representative photographs of slides with weak **(A)**, moderate **(B)**, and strong **(C)** p16 staining intensity.

RESULTS

Patient demographics and clinicopathological findings

The male-to-female ratio was 13:1. A smoking history was available for 83 of the 112 patients, which revealed that 82 (99%) of the 83 cases were smokers, while 1 (1%) was a non-smoker. Alcohol use history was available for 86 patients, with 11 (12%) reporting alcohol consumption, and 75 (87%) were non-drinkers. In the medical history, 10 patients (9%) had a history of secondary malignancy. The most frequent secondary malignancy was lung carcinoma, observed in 4 patients (4%).

Of the 112 patients, 16 (14%) underwent partial laryngectomy, and 96 (85%) underwent total laryngectomy. Neck dissection was performed in 103 (92%) patients. Among the 112 patients, 111 had conventional squamous cell carcinoma, and 1 had adenosquamous carcinoma. The mean survival time of the patients was 4.75 years (minimum 0.3, maximum 11.95, median 4.82). One patient's follow-up data was unavailable and was excluded from the survival analysis. A summary of the demographic and clinicopathological findings, including the stage distribution and treatments received, can be found in Table I.

Table I. Clinicopathological findings of patients

Characteristics	Value
Age (years)	
Mean	62.4
Range	42-88
Gender, n (%)	
Male	104 (92.9)
Female	8 (7.1)
Primary Tumour Site, n (%)	
Supraglottis	37 (33)
Glottis	37 (33)
Subglottis	3 (2.7)
Transglottic	35 (31.3)
T Stage, n (%)	
T1	4 (3.6),
T2	26 (23.2)
T3	48 (42.9)
T4	34 (30.4)
N Stage, n (%)	
Nx	9 (8)
N0	59 (52.7)
N1	9 (8)
N2	24 (21.5)
N3	11 (9.8)
Stage, n (%)	
I	2 (1.8)
II	16 (14.3)
III	36 (32.1)
IV	58 (51.8)
Histopathological Differentiation, n (%)	
Grade I	22 (19.8)
Grade II	65 (58.6)
Grade III	24 (21.6)
Chemotherapy Regimen, n (%) (based on 106 (95%) patients)	
Adjuvant	46 (43.4)
Neoadjuvant	1 (0.9)
Radiotherapy Regimen, n (%) (based on 111 (91%) patients)	
Neoadjuvant	15 (13.5)
Recurrence, n (%)	
Occurred	35 (42.7)
Did not occur	47 (57.3)

p16 Immunohistochemistry Findings

The immunoreactivity of p16 in the cytoplasm and the nucleus of the tumour cells in the tissue microarray blocks was evaluated. p16 immunoreactivity was observed in 18 cases (16%). Of the 18 cases, 2 cases (11% of p16-reactive cases, 1.7% of all cases) the staining was nuclear only. Strong staining of more than 70% of the tumour cells was seen in one case. The p16 IHC score was above 6/9 in 5 cases (28% of p16-reactive cases, 4.4% of all cases). Despite p16 positivity, none of the cases demonstrated transcriptionally active HPV with RT-PCR tests. Additionally, no p16 immunoreactivity was observed in tumour cells of the HPV-positive case. These findings suggest that p16 IHC positivity or a positive p16 IHC score has a sensitivity of 0%, specificity of 99%, and a negative predictive value of 0.99 for detecting transcriptionally active HPV infection in LSCC.

A significant negative impact of the p16 IHC score on survival was observed (Log rank test, $p<0.05$, Figure 2). The relationship with survival was not evident if the criteria of nuclear and cytoplasmic, moderate to strong staining in more than 70% of tumour cells for p16 positivity was used. No statistically significant relationship was found between p16 IHC positivity/p16 IHC score and other factors or outcomes. In the p16-positive case (with more than 70% staining), the HPV mRNA RT-PCR test was negative. This 70-year-old smoker presented with a trans-glottic, grade 3 conventional squamous cell carcinoma and had no metastatic lymph nodes upon neck dissection. This patient remained alive 2.8 years post-surgery.

RT-PCR Analysis Findings

RT-PCR analysis was performed on all 112 cases. One case was excluded from the analysis due to an internal control signal value below the threshold. Among the remaining 111 cases, two cases (1.7%) tested positive for high-risk HPV E6/E7 mRNA targeted amplicon signals. Of the positive cases, one had an analysis/threshold ratio below 0.5 and thus was considered negative. The other case had an analysis/threshold ratio of 2.77 and was considered positive, giving an overall RT-PCR positivity rate of 0.9%. Further subtyping confirmed HPV type 16 E6/E7 mRNA in the positive case (analysis/threshold ratio 2.70). No HPV 18/45 mRNA signals were detected.

The HPV-positive case was a 56-year-old male with a history of 100 pack-years of smoking and alcohol use. He had no known comorbidities and underwent total laryngectomy and bilateral functional neck dissection without pre- or post-operative chemoradiotherapy. Pathological examination revealed a glottic tumour, 2.5 cm in size, with conventional keratinising squamous cell carcinoma morphology (Figure 3). The pathological stage was pT3 with no nodal metastasis; the clinical stage was III. The patient was alive at a survival time of 8.65 years. No p16 staining was observed in tumour cells.

CISH Findings

In CISH tests, FFPE blocks of cervical squamous cell carcinoma with strong and diffuse p16 immunohistochemistry expression and confirmed HPV type 16 positivity on previous cervical smears were used as external controls, and did not show any nuclear or cytoplasmic pigment accumulation compatible with HPV DNA. When the same external

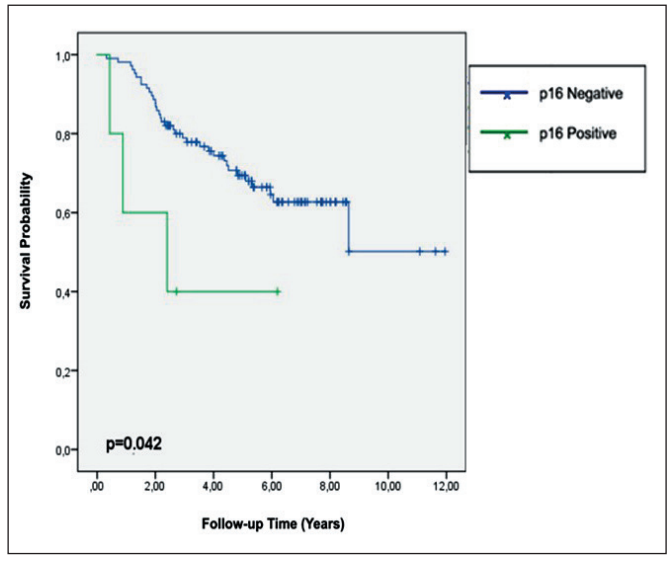


Figure 2: Impact of the p16 immunohistochemistry score on survival (Kaplan-Meier analysis).

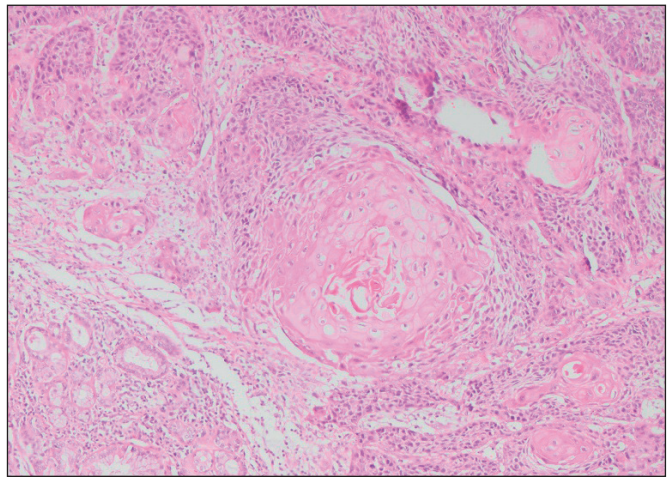


Figure 3: Microscopic photography of an H&E stained slide of the RT-PCR HPV positive case.

control tissue samples were analysed with the "ZytoFast DNA (+) Control Probe" to check nuclear DNA content and viability, strong and diffuse nuclear chromogen accumulation was observed in both tumour and surrounding tissue cells. However, due to the lack of signal in the external control tissues with the ZytoFast HPV High-Risk (HR) Types Probe, the CISH method was not included in further analysis.

DISCUSSION

The main finding of our study is an exceptionally low rate of transcriptionally active HPV infection in LSCC, with only one of 111 evaluable cases (0.9%) positive for HPV 16 E6/E7 mRNA by RT-PCR. This is among the lowest rates reported using mRNA-based methods. Published RT-PCR positivity rates in LSCC range from approximately 1.7% to 6.6% (11–15), and our rate falls below this range. The single HPV-positive patient had a heavy smoking and alcohol history (100 pack-years), meaning that even in the one confirmed case, the relative contribution of HPV versus conventional carcinogens cannot be clearly established. Taken together, the RT-PCR data provide no definite support for a causal role of HPV in LSCC development in our cohort.

Several features of our cohort are consistent with, and help explain, this low HPV prevalence. First, 98.8% of patients with available data were smokers, reflecting a predominantly tobacco-driven aetiology. Second, the morphological distribution of the cases, with no basaloid or non-keratinising variants, mirrors the morphological phenotype of tobacco-associated laryngeal carcinogenesis (16). This absence was not the result of deliberate exclusion but rather reflects the genuine morphological distribution of LSCC in our patient population, and itself contributes to the low overall HPV rate.

The laryngeal mucosa lacks the reticulated crypt epithelium of the tonsillar and base-of-tongue regions, which facilitates HPV uptake and productive infection in the oropharynx (17). This anatomical difference is widely considered to underlie the lower susceptibility of the larynx to HPV-driven carcinogenesis. Additionally, background HPV seroprevalence in Türkiye is reported to be lower than in Western Europe and North America (18), which may further reduce the pool of individuals at risk of HPV-associated laryngeal disease. Two prior Turkish LSCC studies using DNA-based detection methods reported positivity rates that, while higher than ours, were also at the lower end of the global range (19,20). A recent updated meta-analysis of HPV in LSCC estimated a pooled DNA-based prevalence of around 20–28% globally (21,22), with substantial heterogeneity by region and detection method, further contextualising our low mRNA-based rate.

The systematic discrepancy between DNA-based and mRNA-based HPV positivity rates in LSCC is an important interpretive issue. In published series where both methods were applied to the same cohort, RNA PCR positivity was consistently several-fold lower than DNA PCR positivity (19,23). A notable recent example is the 97-case series by Gallus et al. (24), in which HPV 16 DNA was detected in 8.75% of cases yet HPV RNA was undetectable in every case, directly paralleling our 0.9% mRNA-based rate despite higher DNA positivity. This gap likely reflects non-transcriptionally active (latent or passenger) viral DNA present in laryngeal tissue without productive oncogenic infection. Our exclusive reliance on RT-PCR, the current gold standard for confirming transcriptionally active HPV (4,25), therefore yields a more biologically meaningful prevalence estimate than DNA-only studies. The inability of CISH to detect HPV DNA in our series is consistent with the known sensitivity threshold of chromogenic probes when viral copy numbers fall below approximately 25 copies per cell (26,27), and reinforces the appropriateness of RT-PCR as the primary method in our analysis.

Our evaluation of p16 IHC revealed a complete absence of concordance with RT-PCR results. Using the standard 70% diffuse block-positive criterion, one case met the threshold yet was RT-PCR negative. The semi-quantitative scoring approach (IHC score ≥6/9) identified five positive cases, none of which harboured detectable HPV E6/E7 mRNA. The calculated sensitivity of 0% and specificity of 99% for p16 IHC in predicting transcriptionally active HPV demonstrate clearly that p16 IHC cannot serve as a reliable surrogate for HPV status in LSCC — a conclusion supported by prior studies combining p16 IHC with mRNA-based HPV detection (11,12,23,28) and underscored by a 2024 systematic review of p16 in LSCC by Gallus et al. which concluded that the reliability of p16^{INK4a} as a surrogate prognostic marker in LSCC is disproven by the available evidence (29). A 2023 individual patient data analysis by Mehanna et al. (30) — although focused on oropharyngeal SCC — formally quantified the prognostic hazard of p16/HPV discordance, reinforcing that p16-positive but HPV-negative tumours behave biologically like HPV-negative disease, a principle even more applicable in LSCC where HPV-independent p16 overexpression is the norm rather than the exception. In LSCC, where tobacco-driven carcinogenesis predominates, p16 overexpression more likely reflects HPV-independent disruption of the CDKN2A/Rb pathway — such as CDKN2A promoter methylation, copy

number gain, or post-transcriptional deregulation — than viral E7-mediated Rb inactivation (31).

A clinically significant secondary finding was the association between p16 IHC score positivity (score ≥6/9) and decreased overall survival (log-rank $p<0.05$). This contrasts sharply with the well-established favourable prognostic role of p16 in oropharyngeal SCC (8,10) and is consistent with data from LSCC-specific studies reporting worse or neutral outcomes in p16-positive tumours (32,33). In the 97-case Gallus et al. (24) series, p16 overexpression showed a survival benefit in primarily operated cases but an adverse association in salvage surgery patients, highlighting the context-dependence of p16's prognostic value in LSCC and echoing our finding that its relationship with outcomes is not simply favourable. In our HPV-negative context, elevated p16 expression likely reflects biological aggressiveness through Rb pathway derangement rather than viral protection, inverting the prognostic relationship seen in OPSCC. A noteworthy methodological observation is that the 70% block-positive criterion — the threshold borrowed from oropharyngeal SCC guidelines — did not predict survival in our cohort, whereas the semi-quantitative scoring system (IHC score ≥6/9) did. This suggests that graduated quantification of p16 expression captures clinically relevant biological variation that binary thresholds obscure and warrants prospective validation in larger LSCC cohorts (24). The absence of any significant association between p16 IHC positivity and standard clinicopathological parameters suggests that p16's prognostic value in LSCC operates independently of conventional risk stratifiers.

CONCLUSION

In summary, our data demonstrate that transcriptionally active HPV infection plays a negligible role in LSCC carcinogenesis in our population, that p16 IHC is not a reliable HPV surrogate in this anatomical site regardless of the scoring criterion used, and that elevated p16 expression — likely reflecting HPV-independent Rb pathway disruption in this predominantly tobacco-driven cohort — is associated with poorer survival. Future multicentre studies from Turkish centres, utilising concurrent RT-PCR with standardised p16 scoring, would help establish definitive prevalence figures and validate the prognostic utility of quantitative p16 assessment in LSCC.

A limitation of our study warrants acknowledgement. The use of tissue microarray cores for p16 immunohistochemical evaluation has an inherent sampling limitation. Since TMA cores sample only a small area of the tumour (2–3 mm in diameter), they may not fully capture the heterogeneity of p16 expression across the entire tumour section, which is particularly relevant when applying a threshold (34). However, the TMA technique was intentionally employed to maximise the number of cases that could be evaluated within a single study, and this trade-off between sampling depth and case volume should be considered when interpreting the p16 IHC findings.

The very low prevalence of transcriptionally active HPV (n=1) precludes any statistical comparison of HPV-positive versus HPV-negative tumour characteristics, limiting conclusions about the clinicopathological correlates of HPV-positive LSCC in our population. Despite these limitations, the internal consistency of our findings and the use of a gold-standard mRNA-based assay support the reliability of our conclusions.

Funding
This study was funded by the Marmara University Scientific Research Project Coordination Unit (BAP-KO) (grant number 9527).

Conflict of Interest
The authors declare that they have no conflict of interest.

Ethics Statement
Formal consent is not required for this type of study. The study was approved by the Marmara University clinical research ethics board. Informed consent is not required for this type of study.

Authorship Contributions
Concept: **SYE, LC,** Design: **SYE, LC,** Data collection and/or processing: **SYE, LC,** Analysis and/or interpretation: **SYE, LC,** Literature search: **SYE,** Writing: **SYE,** Approval: **SYE, LC.**

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