Different patterns of p16\(^{\text{INK4a}}\) immunohistochemical expression and their biological implications in laryngeal squamous cell carcinoma

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Abstract

Introduction: p16\(^{\text{INK4a}}\) immunohistochemistry (IHC) is widely used to facilitate the diagnosis of human papillomavirus (HPV)-associated neoplasia, when >70% of cells show strong nuclear and cytoplasmic positivity. In this study, we aim to compare partial expression patterns that do not fulfill the above criteria and seek biological implications in laryngeal squamous cell carcinoma (LSCC). Materials and Methods: p16\(^{\text{INK4a}}\) IHC staining was conducted on representative sections of archived tissue from 88 LSCCs. Immunoreactivity was described based on four parameters: intracellular localization of immunostaining, intensity of immunostaining, distribution pattern and percentage of positive cells. Results: Six patterns of p16\(^{\text{INK4a}}\) immunoreexpression were observed and defined as: strong diffuse (strong immunostaining, expression in cytoplasm and nucleus in >70% of tumor cells), weak diffuse (moderate or weak immunostaining, expression in cytoplasm in >70% of tumor cells), marginal (strong cytoplasmic immunostaining, limited to the periphery of tumor islets), strong scattered (strong immunostaining, expression in cytoplasm and nucleus in <50% of tumor cells), weak scattered (moderate or weak immunostaining, expression in cytoplasm in <50% of tumor cells), negative (no expression). The pN stage of the patients was associated with p16\(^{\text{INK4a}}\) immunoreexpression patterns, the marginal pattern was only found in the pN0-Nx stages, while the weak diffuse pattern was more frequently observed in pN2-N3 stages. Conclusions: Partial immunostaining with architecturally distinct p16\(^{\text{INK4a}}\) immunoreexpression patterns may prove significant in stratifying characteristic clinicopathological subgroups among LSCC. Our observations may support the hypothesis that p16\(^{\text{INK4a}}\) has different roles in different subcellular locations, with tumorigenic molecular pathways unrelated to HPV infection.

Keywords: larynx, squamous cell carcinoma, p16, immunohistochemistry.

Introduction

Laryngeal carcinomas are the second most common respiratory tract cancers after lung cancer, thus representing an important global health burden [1]. A marked variation in frequency, both geographical and gender wise, has been noted, squamous cell carcinomas (SCCs) accounting for most histological types [2]. The main risk factors are represented by tobacco and alcohol use. The role of the human papillomavirus (HPV) in the pathogenesis of laryngeal squamous cell carcinoma (LSCC) is still controversial, but research in this area has contributed to a more thorough study of p16\(^{\text{INK4a}}\), whose immunohistochemical expression is used as a surrogate marker for the presence of HPV in cervical and oropharyngeal carcinoma [3–5].

The cell cycle of resting cells is strictly managed through various checkpoints by a set of regulatory proteins. One example is the regulation of G1-to-S progression by two classes of cyclin and cyclin-dependent kinase (CDK) complexes: cyclin Ds–CDK4/6 and cyclin Es–CDK2. These complexes inactivate the retinoblastoma protein (pRB) family through phosphorylation and promote the progression towards mitosis [6]. p16\(^{\text{INK4a}}\) is one of the cyclin–CDK inhibitor proteins; it binds directly to CDK4 and CDK6 and blocks phosphorylation of the pRB, maintaining it in a hypo-phosphorylated and growth-suppressive state and inducing a G1 phase cell cycle arrest [7]. Isolated, characterized, and named in the early 1990’s, this 16 kDa protein is part of the INK4 class of cell cycle inhibitors; the encoding gene, cyclin-dependent kinase inhibitor 2A (CDKN2A) or multiple tumor suppressor 1 (MTS1), has an 8.5 kb length and is located on chromosome 9p21.3 [8, 9]. Considering the role played by the retinoblastoma tumor suppressor (RB) pathways in blocking inappropriate cellular proliferation, the loss of p16\(^{\text{INK4a}}\) and its negative regulator function on the cell cycle is thought to lead to carcinogenesis [10].

The main focus of research for p16\(^{\text{INK4a}}\) immunoreexpression has been the well-established correlation with HPV infection. In the infected cells, the E7 viral oncoprotein functionally inactivates the pRB, and, released from its...
negative feedback control, p16\(^{N\text{Kd4}}\) becomes upregulated, in an attempt to inhibit uncontrolled cellular replication. This leads to an increase of intracellular levels of p16\(^{N\text{Kd4}}\) that can be detected by immunohistochemistry (IHC), rendering this protein an excellent surrogate marker for HPV infections in some settings [11]. In the context of HPV related lesions, there is marked heterogeneity in p16\(^{N\text{Kd4}}\) IHC scoring, different researchers defining positivity based on different combinations of parameters like: the percentage of immunostained cells, immunostaining pattern, and immunostaining intensity. Most practices follow the criteria recommended by the lower anogenital squamous terminology (LAST): a positive result should be considered when there is “block-positivity” (defined as continuous, strong nuclear immunostaining, with or without cytoplasmic signal, with extension from the basal cell layer upward for at least one third of the thickness of the epithelium) [12, 13]. In a tumor sample, “block-positivity” is usually translated as strong nuclear ± cytoplasmic positivity in more than 70% of tumoral cells. Judicious interpretation of the results is advisable regarding cases that are not clear-cut positive or negative, since some ambiguous patterns have been shown to harbor HPV [14, 15].

Various other stressors lead to an aberrant expression of CDKN2A. As a tumor suppressor protein, p16\(^{N\text{Kd4}}\) has been linked to senescence and multiple tumors, like lymphoma, melanoma, odontogenic tumors, pancreatic adenocarcinoma, non-small cell lung cancer, gastrointestinal carcinoma, and prostate cancer, to name just a few [16–18]. Not surprisingly, various models of IHC expression have come to the attention of researchers.

**Aim**

In this study, we aimed to assess the IHC expression patterns of p16\(^{N\text{Kd4}}\) in the setting of LSCC. We focused on partial expression patterns that did not meet the “block-positivity” criteria. Possible biological implications were analyzed using clinicopathological variables and patient outcomes.

**Materials and Methods**

**Tissue specimens**

The material was comprised of formalin-fixed paraffin-embedded (FFPE) tissue samples of laryngeal carcinomas collected in 2009 and 2010. Following a protocol approved by the Medical Ethics Committee of Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania (Approval No. 177/10.05.2016), the Emergency County Hospital Pathology Department’s Database was searched for consecutive patients surgically treated at the Department of Otolaryngology. Tumor samples were searched for consecutive patients surgically treated at the County Hospital Pathology Department’s Database was collected in 2009 and 2010. Following a protocol approved [19]. Tissue specimens

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In addition to the initial evaluation for routine diagnostics, all cases were reviewed by two study pathologists and histologically assessed according to the World Health Organization (WHO) Classification of Head and Neck Tumours (4th edition) [2] recommendations.

**IHC staining**

For each case, representative 3 μm sections of archived FFPE tissue from surgical specimens were placed on silanized glass slides (Dako, Glostrup, Denmark), deparaffinized, and rehydrated through a series of xylene and graded alcohols (100%, 95%, and 75%). Antigen retrieval pretreatment was performed with Novoceastra™ Epitope Retrieval Solution pH 9 (Leica Biosystems, Newcastle upon Tyne, United Kingdom) for 20 minutes at 100°C. p16\(^{N\text{Kd4}}\) IHC staining was conducted using a ready-to-use anti-p16 mouse monoclonal antibody, clone G175-405 (BioGenex, Fremont, CA, USA). Ki67 IHC staining was conducted using anti-Ki67 mouse monoclonal antibody, clone MM1 (Novocastra, Leica Biosystems), used according to the manufacturer’s protocol. DAB was used as chromogen, in order to visualize antigen location sites. Hematoxylin was used for counterstaining. Positive controls consisting of uterine cervix with severe dysplasia (for p16\(^{N\text{Kd4}}\) immunostaining) and tonsil (for Ki67 immunostaining) were included routinely.

**Evaluation of IHC expression**

Two independent investigators analyzed the expression of the tested protein with the use of a DM750 (Leica Biosystems) light microscope coupled with an ICC50 high definition (HD) camera.

\(p16^{N\text{Kd4}}\) immunoreactivity was described following four parameters: intracellular localization of the immunostaining (nuclear, cytoplasmic, both), immunostaining intensity (strong, moderate, mild; positive immunostaining was defined as visual detection of any appreciable shade of DAB beyond the baseline Hematoxylin counterstaining), distribution of positive cell (diffuse – in all layers of tumor islets, limited to the periphery of tumor islets, or scattered as individual cells or as groups of less than 50 contiguous cells demonstrating immunostaining), and the proportion of immunostained tumor cells, semi-quantitatively categorized in increments of 10% by visual estimation. The five most representative 200×-magnification microscopic fields were selected for evaluation.

Based on these parameters, six patterns of p16\(^{N\text{Kd4}}\) immunoexpression were observed and defined as: (i) strong diffuse – strong immunostaining expression in cytoplasm and nucleus in >70% of tumor cells (Figure 1A), (ii) weak diffuse – moderate or weak immunostaining expression in cytoplasm in <70% of tumor cells (Figure 1B), (iii) marginal – strong cytoplasmic immunostaining, limited to the periphery of tumor islets (Figure 1C), (iv) strong scattered – strong immunostaining expression in cytoplasm and nucleus in <50% of tumor cells (Figure 1D), (v) weak scattered – moderate or weak immunostaining expression in cytoplasm in <50% of tumor cells (Figure 1E), and (vi) negative – no expression.
Evaluation of Ki67 immunoreactivity reported the proliferation index, represented by the proportion of tumor cells with nuclear immunostaining in increments of 10%, assessed on 10 400×-magnification microscopic fields, each field containing about 1000 cells.

**Statistical analysis**

Statistical analyses were performed using R with R Commander version 3.6.2. p16INK4a immunoexpression was correlated with baseline clinical and pathological characteristics of the patients, as well as their immuno-profile. Fisher’s exact test of independence was used to analyze categorical variables. Differences between groups were assessed using Mann–Whitney U-test or Kruskal–Wallis test for continuous variables, followed, if significant, by post-hoc analysis for all pairwise comparisons. Shapiro–Wilk test was used to test the assumption of normality.

Overall survival (OS) and disease-free survival (DFS) were calculated using Kaplan–Meier method; OS was measured from date of initial diagnosis to date of death or last follow-up; DFS was calculated from the date of initial diagnosis to that of disease progression, defined as...
cancer recurrence, metastasis, or cancer-related death. Log-rank test was used for analyses evaluating survival by p16\(^{INK4a}\) expression.

\(p\)-value was considered statistically significant if \(<0.05\). When multiple pairwise comparisons were necessary, Bonferroni correction of the \(p\)-values was used in order to control the familywise error rate.

### Results

#### Clinical and pathological features

A total of 88 patients with sufficient tumor samples for p16\(^{INK4a}\) immunostaining were identified and included in this study. All patients were males, with the age range between 45 and 76. Most of the patients had smoking histories, with only nine reported as never smoker. Almost three quarters of the patients admitted to drinking alcohol, habitually or occasionally. Most of the specimens were glottic tumors, less than a third belonging to supraglottic or subglottic subsites. A slight predominance of pT1 stage was observed when analyzing the distribution of cases according to pT stage. Positive lymph node status was noted in 19 cases, five corresponding to pN1 and 14 to pN2 stage; no lymph node metastases were identified in the remaining 69 cases. For 23 out the latter 69 cases, the number of evaluated lymph nodes did not allow accurate pN staging (pNx). Most LSCCs exhibited moderate histological differentiation. SCCs exhibited non-conventional histology in seven (7.95%) cases, the histological subtypes observed along with the conventional SCC being verrucous, basaloid, papillary and spindle cell SCC. Evidence of keratinization was seen in 69 out of 88 cases. Lymph vessel invasion was detected in 20 (22.72%) cases, while blood vessel invasion was detected in seven (7.95%) cases. A minority of cases displayed perineural invasion (five out of 88). Approximately 50% of the patients received radiotherapy/chemoradiotherapy after surgery.

Detailed clinical and pathological characteristics of patients can be seen in Table 1.

| Table 1 – Clinicopathological characteristics by p16\(^{INK4a}\) expression patterns |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Variables       | All patients    | Strong diffuse  | Weak diffuse    | Marginal         | Strong scattered | Weak scattered   | Negative        | \(p\)-value*    |
| Age             | (N=88)          | \((N=3)\)       | \((N=6)\)       | \((N=17)\)       | \((N=3)\)       | \((N=31)\)      | \((N=28)\)      |                |
| Age mean±SD [years] | 58.5±7.8        | 58.3±14.9       | 60.5±9.2        | 58.2±5.3         | 56.6±8.3        | 57.7±7.5        | 59.2±9.9        | 0.929          |
| Smoking         |                 |                 |                 |                 |                 |                 |                 | 0.442          |
| • never smoker  | 9 (10.3)        | 1 (33.3)        | 1 (16.7)        | 2 (11.8)         | 0               | 1 (3.2)         | 4 (14.3)        |                |
| • smoker\(^a\)  | 79 (89.7)       | 2 (66.7)        | 5 (83.3)        | 15 (88.2)        | 3 (100)         | 30 (96.8)       | 24 (85.7)       |                |
| Alcohol use     |                 |                 |                 |                 |                 |                 |                 | 0.435          |
| • no            | 25 (28.4)       | 0               | 1 (16.7)        | 5 (29.4)         | 2 (66.7)        | 11 (35.5)       | 6 (21.4)        |                |
| • yes           | 63 (71.6)       | 3 (100)         | 3 (50)          | 12 (70.6)        | 1 (33.3)        | 20 (64.5)       | 22 (78.6)       |                |
| Anatomical subsite |               |                 |                 |                 |                 |                 |                 | 0.534          |
| • supraglottis  | 20 (22.7)       | 0               | 3 (50)          | 5 (29.4)         | 1 (33.3)        | 6 (19.4)        | 5 (17.8)        |                |
| • glottis       | 64 (72.7)       | 3 (100)         | 3 (50)          | 10 (58.8)        | 2 (66.7)        | 24 (77.4)       | 22 (78.6)       |                |
| • subglottis    | 4 (5.4)         | 0               | 0              | 2 (11.8)         | 0               | 1 (33.2)        | 1 (3.6)         |                |
| T-stage         |                 |                 |                 |                 |                 |                 |                 | 0.279          |
| • pT1           | 31 (35.2)       | 2 (66.7)        | 1 (16.7)        | 8 (47)           | 1 (33.3)        | 8 (25.8)        | 11 (39.3)       |                |
| • pT2           | 11 (12.5)       | 0               | 2 (33.3)        | 2 (11.7)         | 2 (66.7)        | 2 (6.5)         | 3 (10.7)        |                |
| • pT3           | 21 (23.9)       | 0               | 1 (16.7)        | 2 (11.7)         | 0               | 11 (35.5)       | 7 (25)          |                |
| • pT4           | 25 (28.4)       | 1 (33.3)        | 2 (33.3)        | 5 (29.4)         | 0               | 10 (32.2)       | 7 (25)          |                |
| N-stage         |                 |                 |                 |                 |                 |                 |                 | 0.004*         |
| • pNx           | 23 (26.1)       | 2 (66.7)        | 1 (16.7)        | 3 (17.6)         | 1 (33.3)        | 7 (22.6)        | 9 (32.1)        |                |
| • pN0           | 46 (52.3)       | 1 (33.3)        | 1 (16.7)        | 14 (82.4)        | 2 (66.7)        | 15 (48.4)       | 13 (46.4)       |                |
| • pN1-N2        | 19 (21.6)       | 0               | 4 (66.6)        | 0               | 0               | 9 (29)          | 6 (21.5)        |                |
| Differentiation |                 |                 |                 |                 |                 |                 |                 | 0.073          |
| • well          | 17 (19.3)       | 0               | 0              | 4 (23.5)         | 1 (33.3)        | 4 (12.9)        | 8 (28.6)        |                |
| • moderate      | 47 (53.4)       | 3 (100)         | 3 (100)         | 10 (58.8)        | 1 (33.3)        | 13 (41.9)       | 17 (60.7)       |                |
| • poor          | 24 (27.3)       | 0               | 0              | 3 (17.7)         | 1 (33.3)        | 14 (45.2)       | 3 (10.7)        |                |
| Histological subtype |             |                 |                 |                 |                 |                 |                 | 0.356          |
| • conventional  | 81 (92)         | 3 (100)         | 4 (66.6)        | 17 (100)         | 3 (100)         | 28 (90.4)       | 26 (92.8)       |                |
| • verrucous     | 1 (1.1)         | 0               | 0              | 0               | 0               | 1 (3.2)         | 0               |                |
| • basaloid      | 2 (2.3)         | 0               | 0              | 0               | 0               | 1 (3.2)         | 1 (3.6)         |                |
| • papillary     | 1 (1.1)         | 0               | 1 (16.7)       | 0               | 0               | 0               | 0               |                |
| • spindle cell  | 3 (3.5)         | 0               | 1 (16.7)       | 0               | 0               | 1 (3.2)         | 1 (3.6)         |                |
Median follow-up time for the patients was 5.66 years (with a maximum of 7.28 years). During follow-up, 34 deaths occurred. In the full cohort, the 5-year OS was 63.6% [95% confidence interval (CI): 54.3%–75.5%], and the 5-year DFS was 51.1% (95% CI: 41.7%–62.7%).

**IHC assessment**

IHC study showed positivity to p16\(^{\text{INK4a}}\) in 68.18% (\(n=60\)) of the samples. Overall, three (3.41%) patients had a strong diffuse pattern of p16\(^{\text{INK4a}}\) immunoeexpression, six (6.82%) patients had a weak diffuse immunostaining pattern, 17 (19.32%) patients had a marginal immunostaining pattern, three (3.41%) patients had a strong scattered immunostaining pattern, and 31 (35.23%) patients had a weak scattered immunostaining pattern. p16\(^{\text{INK4a}}\) was negative in the remaining 28 (31.82%) cases.

In order to compare the partial p16\(^{\text{INK4a}}\) immunoeexpression patterns that do not meet the classical HPV-related positivity criteria (more than 70% of the cells demonstrate intense nuclear and cytoplasmic positivity) and to analyze their potential biological implications in LSCC, statistical analyses were performed without the strong diffuse group.

Variables such as age, smoking, alcohol consumption, anatomical site, histological subtype, keratinization, lymphovascular and perineural invasion, or pT stage did not show significant differences for p16\(^{\text{INK4a}}\) immunoeexpression patterns. However, there was a statistically significant association between the p16\(^{\text{INK4a}}\) immunostaining groups and the pN staging. The marginal pattern was only found in the pN0 stages and cases in which lymph nodes were not harvested due to no clinical suspicion of lymph node metastasis (pNx). To avoid misinterpretation of the real nodal status, further analysis of the relation between p16\(^{\text{INK4a}}\) immunostaining groups and the pN staging disregarded the pNx cases (Table 1).

**Post-hoc** test comparing the group of tumors with marginal immunoeexpression pattern to the other immunoeexpression patterns, by pN stage did not show significant differences for p16\(^{\text{INK4a}}\) immunoeexpression patterns. However, there was a statistically significant association between the p16\(^{\text{INK4a}}\) immunostaining groups and the pN staging. The marginal pattern was only found in the pN0 stages and cases in which lymph nodes were not harvested due to no clinical suspicion of lymph node metastasis (pNx). To avoid misinterpretation of the real nodal status, further analysis of the relation between p16\(^{\text{INK4a}}\) immunostaining groups and the pN staging disregarded the pNx cases (Table 1).

**Figure 2 – Distribution of cases with marginal immunoeexpression pattern versus other patterns, by pN stage.**

**Table 2 – Pairwise comparisons of p16\(^{\text{INK4a}}\) expression patterns by pN stage.**

| Compared patterns                  | p-value* |
|------------------------------------|----------|
| Marginal vs Negative               | 0.027    |
| Marginal vs Weak scattered          | 0.014    |
| Marginal vs Strong scattered        | –        |
| Marginal vs Weak diffuse            | 0.001    |
| Negative vs Weak scattered          | 0.755    |
| Negative vs Strong scattered        | 1        |
| Negative vs Weak diffuse            | 0.122    |
| Strong scattered vs Weak scattered  | 0.529    |
| Strong scattered vs Weak diffuse    | 0.142    |
| Weak scattered vs Weak diffuse      | 0.143    |

*Significant p-value after Bonferroni correction is \(p<0.005\).

The Ki67 immunostaining showed positivity in 86 out of 88 cases. In well-differentiated tumors, the average Ki67 proliferation index was 17.05±6.85%. Poorer histological differentiation was associated with a significantly higher Ki67 proliferation index (\(p<0.001\), Kruskal–Wallis test), with average values of 24.77±11.51% for moderately differentiated carcinoma and 31.66±12.74% for poorly differentiated forms (Figure 3). The proportion of Ki67-positive cells was significantly higher in advanced stages (\(p<0.001\), Kruskal–Wallis test), values rising from 18.62±
7.89% and 23.63±12.86% for pT1 and pT2 tumors to 27.14±10.55% and 32.08±13.50% for pT3 and pT4 tumors. The same trend was observed regarding pN stages (p<0.001, Kruskal–Wallis test), with average values of Ki67 proliferation index ranging from 22.66±11.75% in pN0 cases to 37.14±10.69% in pN2 cases. There was no statistically significant difference between average values of Ki67 proliferation index depending on anatomical site, smoking habits, lymphovascular or perineural invasion (all p-values >0.05 on Kruskal–Wallis U-test).

When investigating the IHC expression of Ki67 in the described p16INK4a immunoexpression patterns, the mean values of Ki67 proliferation index found to be significantly heterogeneous among groups (p<0.001, Kruskal–Wallis test). The average value of Ki67 proliferation index was lowest in the marginal immunostaining pattern group (11.76±5.28%). Tumors with weak p16INK4a IHC expression, like the weak diffuse and the weak scattered immunostaining pattern groups had significantly higher values of the Ki67 proliferation index (48.33±7.52% and 30.64±8.92%) (Figure 4).

In OS and DSF analyses, p16INK4a immunoexpression patterns showed no significance, neither in the general cohort (p=0.117, respectively p=0.094, log-rank test), nor in the radiotherapy/chemotherapy group (p=0.89, respectively p=0.186, log-rank test).

**Discussions**

This study is focused on equivocal p16INK4a IHC expression patterns in LSCC. We revealed a significant association between p16INK4a immunoexpression and both pN staging and Ki67 proliferation index.

The conventionally accepted criteria for p16INK4a IHC positivity (strong, diffuse nuclear ± cytoplasmic immunoexpression observed in >70% of tumoral cells) implies that all other staining patterns are to be scored negative or regarded as not related to HPV infection. This consensus, however, has been set by studies on oropharyngeal, cervical, and anal carcinomas. p16INK4a immunoexpression and its distinct architectural morphologies in non-oropharyngeal head and neck squamous cell carcinoma (HNSCC) are yet to be fully evaluated [19].

Although deemed by most studies as nonspecific, we considered the cytoplasmic localization of p16INK4a as positive and included it among the described parameters. Ultrastructural studies have suggested it should not be ignored: electron microscopy evidence of its specific cytoplasmic immunolocalization has been published [20], and subcellular fractionation confirmed that the nuclear and cytoplasmic fractions seem to be the same [21]. Moreover, both Zhao et al. [22] and Lai et al. [23] hypothesized that nuclear and cytoplasmic p16INK4a immunoexpression have different prognostic implications in HNSCC and respectively, oropharyngeal carcinoma. Possible explanations for the underlying mechanism leading to cytoplasmic accumulation of p16INK4a vary from damage of the cytoplasmic–nucleus shuttling in a manner similar to that reported for breast cancer 1 (BRCA1), to it representing a means of inactivating p16INK4a or a defect protein localized to the cytoplasm secondary to mutations of p16 gene [20, 21].

Different immunolabeling patterns of p16INK4a have been studied mainly on HNSCC as a group, most studies performing tests on tumor samples from various anatomical sites, with no studies focused on the larynx, to our knowledge. Variations in histological characteristics of SCC in different anatomical sites might correlate with p16INK4a immunoexpression patterns; numerous studies have indeed underlined the association of oropharyngeal basaloid or nonkeratinizing SCC (distinct features of HPV+ tumors) with a strong, diffuse p16INK4a positivity. Moreover, this correlation has been incorporated in the most recent Tumor, Node, Metastasis (TNM) staging system for HNSCC [24]. But this tight relation between HPV and p16INK4a is not found in LSCC, an important percentage of these tumors being HPV-/p16+ or HPV+/p16−. Further proof that the protein’s increased expression is not completely specific to pRB-E7 oncoprotein pathway [25].

Our study identified four patterns of p16INK4a immunoexpression: marginal, weak diffuse, strong scattered, weak scattered, alongside the strong diffuse and negative cases. Similar to previous studies [26, 27], we noted a low frequency of the strong diffuse pattern (conventional positivity) in our cohort. This low proportion of p16INK4a positive LSCC cases has been reported even by authors...
that chose to use a less stringent cutoff for $p16^{\text{NK4a}}$ positivity, like 30% [27].

Attempting to correlate $p16^{\text{NK4a}}$ with cancer immunity, Ryu et al. [19] analyzed the architectural patterns of IHC expression in HNSCC, identifying five distinct types of immunostaining: strong, marginal, mosaic, nuclear, and absent. As expected, the strong pattern (conventional positivity) was most frequently noted for the oropharynx, with less than 10% of laryngeal cases showing this type of immunoexpression. In contrast to our study, the marginal pattern, which was defined as partial staining restricted to tumor margins or tumor buds, without specifying intra-cellular localization, was not observed in LSCC, but only for oral cavity SCC. This might be explained by the smaller number of LSCC included in the study – 42, as opposed to the 202 oral cavity SCC. Kindred categories were used by Chen et al. [28] to describe equivocal $p16^{\text{NK4a}}$ immunostaining in HNSCC: isolated cells at periphery of nests, faint-diffuse, faint-isolated, and faint-diffuse with patches of strong immunostaining. Chen et al.’s group specifies that the first category, that of isolated cells located at the periphery of the nests, have membranous/cytoplasmic staining. Unfortunately, there is no mention of the tumor site relative to the immunostaining pattern, so we do not know the immunostaining status of the five laryngeal samples their study included.

Other systems used when addressing partial $p16^{\text{NK4a}}$ immunostaining in HNSCC include employing a composite score based on immunostaining intensity and the percentage of positive tumor cells for cytoplasm and nucleus separately [22, 23] or based on immunostaining intensity, percentage of positive tumor cells, and confluence of immunostaining (groups of more than 10 tumor cells), both nuclear and cytoplasmic immunoexpression needed for the cell to be considered positive [29, 30]. Out of the previously mentioned studies, only the one conducted by Zhao et al. included LSCC cases in the research cohort, most LSCCs belonging to a low immunostaining group.

The marginal pattern that both we and Ryu et al. noted has been previously described in basal cell carcinoma and squamous cell carcinoma of the skin, as $p16^{\text{NK4a}}$ immunoreactivity mainly seen in cells located at the edges of tumor nodules [31, 32]. Being overexpressed at the infiltrative front, it has been suggested that it is involved in tumor invasion, with a common functional role in both cutaneous tumors [17]. The growth of neoplasms might be limited by the tumor suppressive mechanism the protein is part of [33], but $p16^{\text{NK4a}}$’s involvement in regulating infiltrative behavior seems to be independent of pRB, since phosphorylated pRB has been observed in the same marginal location in tumors that had $p16^{\text{NK4a}}$ immunoexpression only in the cytoplasm. So, the protein was upregulated at the invasive front even though it was not exerting its function as an inhibitor of proliferation via the pRB pathway [34].

We found a significant association between $p16^{\text{NK4a}}$ immunoexpression patterns and pN staging, with all marginal pattern cases belonging to the pNx-0 group. The result is in partial concordance with Ryu et al.’s [19] findings of a significant association of $p16^{\text{NK4a}}$ immunoexpression patterns with pN staging; in their study, the percentage of cases showing a marginal immunoexpression pattern was two times higher in the pN0-1 group than in the pN2-3 one. The presence of the marginal immunostaining pattern solely in cases with no lymph node involvement suggests that $p16^{\text{NK4a}}$ might have a role in regulating tumor spread. Additionally, similar to the findings reported by Zhao et al., we also noted that patients with the weak diffuse pattern had a higher nodal stage (pN2) [22]. On the other hand, our study found no significant differences for $p16^{\text{NK4a}}$ expression patterns depending on whether lymphovascular invasion was present, thus the mechanism underlying the relation between $p16^{\text{NK4a}}$ immunoexpression at the periphery of tumor nests and lymph node metastasis remains an open question.

$p16^{\text{NK4a}}$ immunoexpression patterns were also found to be associated with Ki67 proliferation index in our study. Tumors with $p16^{\text{NK4a}}$ overexpressed at the periphery of the tumor nodules had a lower Ki67 proliferation index. Through $p16^{\text{NK4a}}$ and Ki67 double immunostaining, Svensson et al. revealed ceased proliferation at the invasive front of the tumor nodules that expressed up-regulated $p16^{\text{NK4a}}$ at the edge of the nodules [31]. Our observation regarding the increased Ki67 proliferation index in tumors that exhibited a weak scattered or a weak diffuse immuno-staining pattern is in concordance with Nilsson et al.’s work on cutaneous SCC, who noted an overlap between weak cytoplasmic $p16^{\text{NK4a}}$ immunoexpression and presence of proliferation [34]. Also, the elevation of Ki67 proliferation index along with tumor dedifferentiation and stage that we detailed has been described by previous studies, like the one published by Ciesielska et al. Moreover, they revealed a moderate positive correlation between Ki67 nuclear expression and $p16^{\text{NK4a}}$ cytoplasmic expression in cancer cells [35].

Whereas previous studies have shown a prognostic significance of cellular localization in HNSCC [19, 22, 23], such an association was not found in our study, neither for OS nor for DFS. The apparent lack of consensus between our findings and other studies can be explained by the fact that the aforementioned studies have included cases with strong diffuse nuclear and cytoplasmic positivity in their statistical analyses, while we have only analyzed cases with equivocal staining. Also, as we pointed out earlier, previous findings refer to HNSCC as a group, including oropharyngeal tumors, and a better outcome of $p16^{\text{NK4a}}$ positive oropharyngeal tumors has been proven [36–38]. Low expression of CDKN2A messenger ribonucleic acid (mRNA) and $p16^{\text{NK4a}}$ were associated with a poor clinical prognosis independently of other known prognostic factors in HPV- HNSCC [39]. Likewise, it is believed that the overexpressed $p16^{\text{NK4a}}$ found in HPV+ HNSCC might be mechanistically involved in their radiosensitivity, hypothesis that has been tested by effectively radiosensitizing HPV- cells by means of CDK4/6 inhibition using Palbociclib [40]. For the minority of $p16^{\text{NK4a}}$ overexpressing LSCC, no prognostic significance was found even in highly homogeneous patient material, like that used by Tiefenböck-Hansson et al. (pT2-3N0 glottic SCC treated with radiotherapy/chemo-radiotherapy) [41]. Similarly, we did not find differences related to $p16^{\text{NK4a}}$ staining patterns in our subgroup of patients that underwent radiotherapy/chemoradiotherapy.
Smoking-related cancers can express p16INK4a, frequently altered in function. Tobacco exposure can entail various epigenetic changes leading to aberrant p16INK4a immunoexpression, with low intensity immunostaining or abnormal localization [23]. We failed to find an association between immunoexpression patterns and smoking habits. A possible explanation lies in López et al.’s work on a cohort of smokers with HPV- LSCC; even though almost all samples presented losses at 9p21 segment, the most common finding being a small deletion of the p16INK4a locus, overall, they found no correlation between protein expression and gene status [42].

The most significant strength of this study is the cohort consisting of a single site of HNSCC, namely the larynx. As previously mentioned, HNSCC of different subsites have been frequently grouped together. Considering the different clinical features and risk factors, this praxis needs to be reviewed, with more studies conducted on single site large cohorts. This study also has certain limitations. Because of its retrospective nature, patients are heterogeneous in stage, treatment, and other factors that might impact our findings. Also, it made quantitative assessment of tobacco exposure unachievable. Since both duration and intensity of smoking were associated with worse survival in patients with p16-positive oropharyngeal tumors [43], grouping patients as nonsmokers or current smokers and collective evaluation, is dissatisfying. Also, there are inherent limitations in the subjectivity of IHC staining interpretation due to expression heterogeneity and due to immunostaining intensity of the particular clone we used. Comparing the G175-405 clone to the E6H4 clone, both from Ventana Medical Systems, Barasch et al. [30] found the G175-405 clone may be more susceptible to partial reactivity, showing weaker immunostaining, especially in the nuclei of tumor cells. Final determination of p16INK4a status, however, showed concordance between the two clones. Lastly, clarifying the link between HPV infection and partial p16INK4a immunoexpression demands that HPV infection status should be evaluated. HPV status was not determined in our study, and this may be a limitation of our findings. Nevertheless, we aimed to provide insights into p16INK4a beyond its role as an immunomarker for HPV.

Human cancers have been reported to harbor mutation, promoter methylation, or deletion of the ARF/INK4a locus, with a frequency second only to p53 gene mutations [33]. Such frequency in alterations justifies research into targeted therapeutic approaches that could be designed around targeting p16INK4a. On one hand, therapies that restore p16INK4a by demethylation or inhibition of cytoplasmic p16INK4a sequestering proteins could lead to cancer elimination through premature senescence induction in tumors in which p16INK4a has a low expression or aberrant subcellular location [32]. On the other hand, patients with p16INK4a-overexpressing tumors, currently resistant to available compounds with specificity against p16INK4a, positive cells, could benefit from gene therapy that allows specific elimination of p16INK4a-overexpressing cells [44]. Although gene delivery is still neither safe nor feasible in humans, progress in anti-cancer interventions means that accurate knowledge of p16INK4a expression is fundamental for guiding research into new therapies.

**Conclusions**

This study reaffirms that strong positivity in p16INK4a immunostaining appears in a minority of LSCC. Equivocal immunostaining shows several architectural patterns that may prove significant in stratifying characteristic clinicopathological subgroups among LSCC, particularly referring to nodal involvement. Therefore, evaluating p16INK4a immunohistochemistry both with conventional quantitative criteria and by architectural pattern could be of value. Cytoplasmic localization of p16INK4a may represent a part of a different modulating pathway, not just a way to inactivate p16INK4a’s cell cycle control function, and might be involved in tumorigenic molecular pathways probably unrelated to HPV infection.

**Conflict of interests**

The authors declare that they have no conflict of interests.

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