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RESEARCH

Differential Biomarker Expression in Head and Neck Cancer Correlates with Anatomical Localization

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Abstract We tested the expression of known ($p16^{ink4}$, Ki67, p53, EGFR) and a new immunohistochemical (collagen XVII/BP180) biomarker in head and neck squamous cell carcinomas (SCC) of diverse anatomical localization. Tissue microarrays (TMA) of 124 SCC were created, immunostained, and analyzed following whole slide digitalization using the Pannoramic Scan and the TMA Module software (3DHISTECH Kft, Budapest, Hungary). Statistical analysis of scoring results was carried out using Pearson's chi-square test. We observed the significant elevation of $p16^{ink4}$ and Ki67 expression in supraglottic, tonsillar and tonsillo-lingual SCCs compared to those affecting the oral cavity, oropharynx without tonsils, larynx without supraglottis and the hypopharynx. This differential antigen expression may reflect the diverse route of embryologic differentiation followed by the affected regions except those of the tonsils and the supraglottis which show similar antigenic pattern but diverse developmental path. All the other biomarkers tested including p53, collagen XVII and EGFR were detected in the majority of cancers including high grade cases, but did not reveal any significant regional difference. Based on our results oropharyngeal squamous cell carcinomas may not be regarded as one entity. Concerning the oral cavity and the oropharynx, cancers affecting the tonsils (palatine and lingual) show significantly elevated $p16^{ink4}$ and Ki67 expression; so as the cancers of the supraglottis compared to the rest of larynx. Consequently, tonsillar and supraglottic cancers show similar biomarker profiles. Correlation of differential biomarker expression with diverse biological behavior in head and neck cancers need further investigations.

Keywords Biomarker expression · Head and neck squamous cell carcinoma · Anatomical localization related pattern · $p16^{ink4}$ · Ki67 · Collagen XVII · p53 · EGFR

Abbreviations

Cdk	Cyclin-dependent kinases
CollXVII	Collagen XVII/BP 180
DAB	Diamino-benzidine
EDTA	Ethylene diamino tetraacetic acid
EGFR	Epidermal growth factor
HNSCC	Head and Neck squamous cell carcinoma
HPV	Humanpapilloma virus
Rb	Retinoblastoma protein
SCC	Squamous cell carcinoma
TBS	Tris buffered saline
TMA	Tissue microarray

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Introduction

Although the vast majority of head and neck cancers is squamous cell carcinoma (SCC), SCC affecting the oral cavity, oropharynx, hypopharynx or the larynx can substantially differ regarding the invasiveness, growth rate and metastatic capacity of these tumors [1, 2]. Molecular genetic differences in these tissue regions possibly play a role in this respect. Recent reports show that HPV (humanpapilloma virus) positive oropharyngeal SCC represents a distinct clinicopathological entity associated with better prognosis compared to HPV negative oropharyngeal SCC [3–5]. In HPV positive tumors, the viral protein E7 binds to retinoblastoma susceptibility protein (pRb), causing rapid degradation of pRb and increased expression of $p16^{ink4}$, a cyclin-dependent kinase inhibitor through a feedback interaction [6–8]. The increased expression of $p16^{ink4}$ in these oropharyngeal carcinomas is relatively easy to detect and has been shown to be an excellent surrogate marker of biologically active HPV infection [3, 9, 10]. In this study, we systematically tested the expressions of $p16^{ink4}$ and some other published prognostic markers (Ki67, p53, EGFR) along with a recently discovered early marker of SCC: Collagen XVII/BP 180 (CollXVII) [11] in head and neck cancers of different anatomical regions using tissue microarrays (TMA) and immunohistochemistry. Our goal was to clarify the regional differences in the expression of potential biomarkers of SSC progression in the head and neck region, which may assist in understanding of the diverse behavior of these malignancies.

Materials and Methods

Patients

124 head and neck cancer patients (27 females, 97 males, average age: 59 years, range from 41 to 89 years) were selected from the archives of the Oto-Rhino-Laryngology, Head and Neck Surgery Department and Pathology Department of Jahn Ferenc South-Pest Hospital, Budapest, Hungary. Tumors of the oral cavity, oropharynx, hypopharynx, larynx were distinguished. Regarding the histological type, all neoplasias were SCCs. As for primary treatment, the patients were given surgical therapy.

Immunohistochemistry

The immunohistochemical examinations were performed in the 1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary. Five blocks of 70-sample tissue microarrays (350 samples) of 2 mm core diameter were created from the tissue samples with the computer controlled TMA Master (3DHISTECH Kft, Budapest, Hungary) instrument. Immunohistochemical staining was performed on 4 μm thick TMA sections using the NovoLink (Leica-NovoCastra, Newcastly-Upon Tyne, UK) polymer detection kit. First, the slides were dewaxed in xilene and rehydrated in ethanol series. Endogenous peroxidase activity was inhibited in 3% hydrogen peroxide for 20 min. Antigen unmasking was done in Tris-EDTA buffer pH 9.0 by an electric pressure cooker at ~105°C for 30 min. The samples were consecutively treated with: protein block for 20 min; the optimally diluted primary antibodies overnight (Table 1); the post-primary reagent for 30 min., and finally with the NovoLoink-peroxidase polymer for 30 min. Then the slides were washed between all incubation steps 2×5 min in Tris-buffered saline pH 7.4 (TBS). The peroxidase activity was visualized with DAB/H_2O_2 for 5–10 min as described by the manufactures. Nuclear counterstaining was done with hematoxylin-eosin. All of the incubations were performed in humid chambers at room temperature.

Immunostained TMA spots were analyzed and scored following full-slide digitalization with the Pannoramic Scan and the database-linked TMA Modul software (both 3DHISTECH) (Fig. 1).

Table 1 Specification of primary antibodies and the conditions of their use

Antigen	Source clone (code)	Vendor	Specificity	Localization	Dilution
Collagen XVII	M-mono 9G2 (6D1) for aa507-529	Homemade [11]	Transmembrane collagen-matrix anchorage	Cytoplasmic (cell membrane)	1:200
Ki67	M-mono Mib1	DAKO	cell-proliferation (G1-M phases)	Nuclear	1:1 (RTU)
MCM2	M-mono CRCT2.1	LabVision-Thermo	Minichormosome maintanance,—cell cycle licensing	Nuclear	1:200
$p16^{ink4}$	M-mono JC8	Lab Vision-Thermo	Cyclin dependent kinase inhibitor of the INK4 family	Nuclear (cytoplasmic)	1:400
EGFR	M-mono 3C6	Ventana-Roche	Epidermal growth factor receptor	Cell membrane (cytoplasmic)	1:1 (RTU)

Vendors' specifications: Dako Ltd. (Glostrup, Denmark), LabVision-Thermo Fisher Scientific Inc. (Fremount, CA, USA); Ventana-Roche (Tucson, AZ, USA)

M-mono Mouse monoclonal

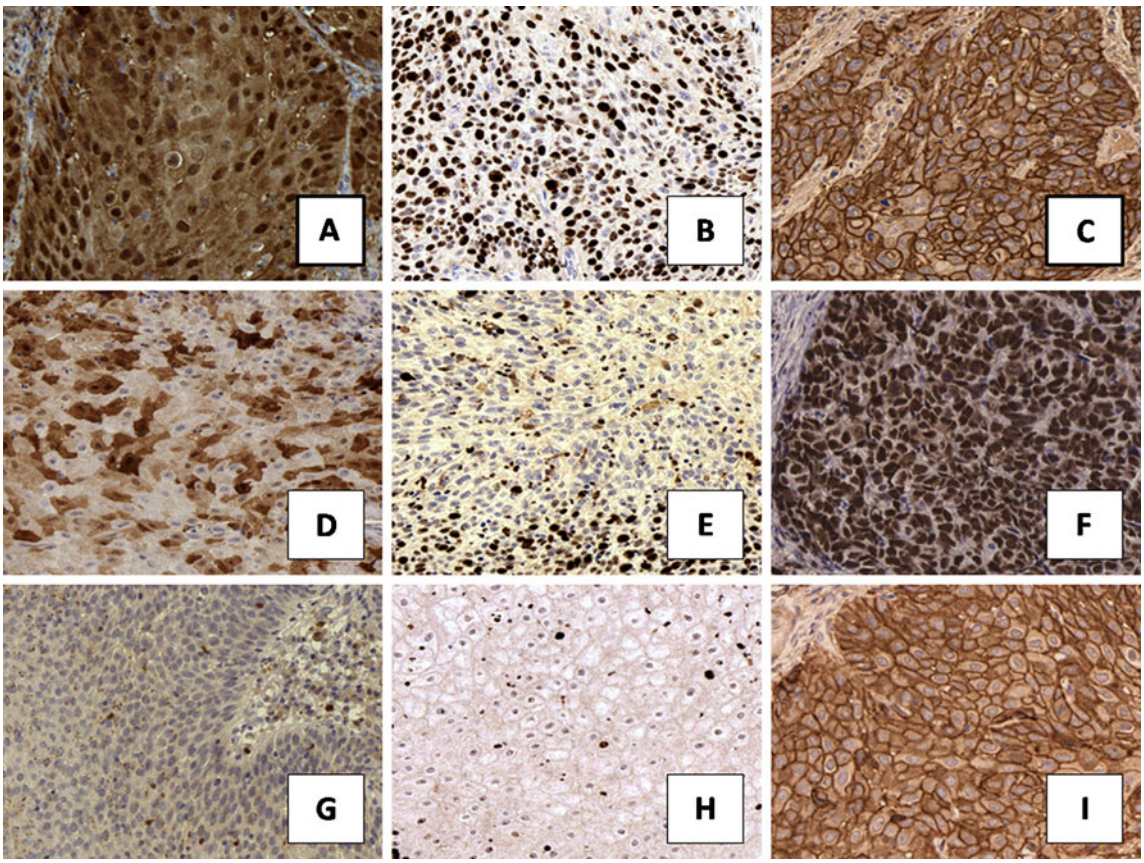

Fig. 1 Representative immunostaining results in head and neck squamous cell carcinoma. Magnification x200. **a** Strong $p16^{ink4}$ staining in an SCC from the tonsillar region. **b** Strong Ki67 staining in an SCC from the supraglottic region. **c** Strong CollagenXVII staining in an SCC from the glottic region. **d** Moderate $p16^{ink4}$ staining in an SCC from the supraglottic region. **e** Moderate Ki67 staining in an SCC from the tonsillar region. **f** Strong p53 staining in an SCC from the oral cavity region. **g** Weak $p16^{ink4}$ staining in an SCC from the transglottic region. **h** Weak Ki67 staining in an SCC from the glottic region. **i** Strong EGFR staining in an SCC from hypopharyngeal region

Scoring was performed by three independent assessors using a 4-grade system and scoring results were correlated with anatomical location and statistically analyzed. At least 3 tumor nests of >200 cells were considered for scoring. The meaning of the scores was the following: Score 0: <5% weak staining; score 1: <25% positive tumor cells, score 2: 25–50% positive tumor cells; and score 3: >50% positive tumor cells. At statistical analysis scores 0 and 1 were considered as negative, while 2 and 3 as positive.

Statistical Analysis

Multivariance analysis using the Pearson's chi-square test was used to determine the probability of differential antigen expression between studied groups of head and neck neoplasias. The statistical analysis was carried out using BMDP Statistical Software, Inc. (Los Angeles, California, USA), and significant difference was declared when the *P* value was <0.05.

Results

Detailed results of the immunohistochemical staining are summarized in Table 2., and shown according to anatomical localizations in Fig. 2. The Neoplastic lesions included SCC of the oral cavity—7 cases (tongue, sublingual region); oropharynx—25 cases (5 tonsil, 7 tonsilo-lingual, 3 soft palate, 10 other part of oropharynx); hypopharynx—13 cases; larynx—79 cases (25 supraglottic, 38 glottic, 16 transglottic). The ratio of positive cases for each biomarker is summed up into the main anatomical regions (Table 3.). $P16^{ink4}$ positivity was detected: oral cavity 42.9%, oropharynx 60%, hypopharynx 53.8%, larynx 48.1% (p=0.7287). Ki67 positivity was found: oral cavity 42.9%, oropharynx 76%, hypopharynx 84.6%, larynx 59.5% (p=0.1097). Collagen XVII positivity was the following: oral cavity 85.7%, oropharynx 64%, hypopharynx 76.9%, larynx 78.5% (p=0.4589). P53 positivity: oral cavity 71.4%, oropharynx 60%, hypopharynx 61.5%, larynx 53.2% (there was no significant difference between the four groups, p=0.7409).

Table 2 Ratio of head and neck cancers positive for the detected biomarkers according to studied regions and sub-regions

Region	Subregion	n	$P16^{ink4}$ pos.	Ki67 pos.	Coll XVII pos.	P53 pos.	EGFR pos.
Oral cavity n=7	ling + subling.	7	3 (42.8%)	3 (42.8%)	6 (85.7%)	5 (71.4%)	7 (100%)
Oropharynx n=25	Total		15 (60%)	19 (76%)	16 (64%)	15 (60%)	18 (72%)
	Soft palate	3	2 (65%)	2 (65%)	2 (65%)	1 (33%)	2 (65%)
	Oropharynx (other)	10	4 (40%)	5 (50%)	3 (30%)	6 (60%)	5 (50%)
	Tonsil	5	3 (60%)	5 (100%)	4 (80%)	2 (40%)	5 (100%)
	Tonsillo-lingual	7	6 (86%)	7 (100%)	7 (100%)	6 (86%)	6 (86%)
Hypopharynx n=13		13	7 (53.8%)	11 (84.6%)	10 (77%)	8 (61.5%)	11 (84.6%)
Larynx n=79	Total		38 (48%)	47 (59%)	62 (78.5%)	42 (53%)	65 (82%)
	Supraglottic	25	18 (72%)	20 (80%)	20 (80%)	14 (56%)	24 (96%)
	Glottic	38	15 (39%)	15 (39%)	30 (77%)	21 (55%)	29 (76%)
	Transglottic	16	5 (31%)	12 (75%)	12 (75%)	7 (44%)	12 (75%)
Total n=124							

EGFR positivity: oral cavity 100%, oropharynx 72%, hypopharynx 84.6%, larynx 82.3% (p=0.3626). Since none of the detected differences in biomarker expression showed significant regional pattern, some of the subregions were highlighted and compared with others. This concept was mainly based on the distinct developmental routes followed by the selected subregions.

When we considered the tonsillar tumors (tumors of the lingual tonsil, tonsillo-lingual region and palatine tonsil) together, and separated them from those of the oropharyngeal region, to compare the frequency of positive cases between these groups, we found considerable differences for some markers as summarized in Table 4.

The $p16^{ink4}$ positivity of tonsillar tumors (75%) was higher than that of the oral cavity + other oropharynx (45%) but the difference was not significant (p=0.0977). The Ki67 positivity (100%) was significantly higher (p=0.0031) in tonsillar tumors than in the tumors of oral cavity including other oropharynx subregions (50%). The ratio of collXVII positive tonsillar tumors (91.7%) was remarkably higher than that of the oral cavity + other oropharyngeal tumors (65%), but it was not significant (p=0.0917). On the other hand, p53 positivity was 66.7% in the tonsillar, and nearly the same (60%) in the oral cavity + oropharynx tumors(p= 0.7061), while EGFR positivity was also somewhat elevated in tonsillar (91.7%), compared to the oral cavity + oropharyngeal (70%) SCC (p=0.1512).

SCC of supraglottis was also separated from other those affecting the larynx (glottis and transglottis) to hypopharynx. The detected frequency of biomarker expression in these regions is summarized in Table 5. The $p16^{ink4}$ positivity of supraglottic tumors (72%) was significantly higher (p=0.0142) than the positivity of other laryngeal (37%) and hypopharyngeal tumors (53,8%). Also, the number of Ki67 positive supraglottic tumors (80%) was significantly higher (p=0.0081) than the positivity of other

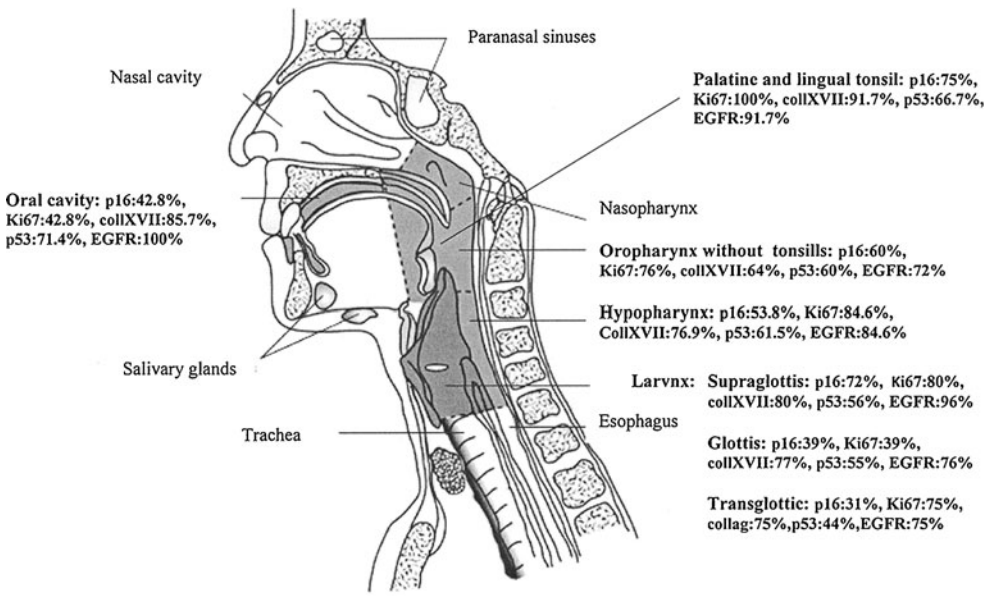

Fig. 2 Detailed results of the immunohistochemical staining ($p16^{ink4}$, Ki67, collXVII, p53, EGFR) according to anatomical localizations

Table 3 Summary of head and neck cancers shown in Table 2 concerning the ration of positive cases in the studied regions

Localization ⇓	$p16^{ink4}$ pos.	Ki67 pos.	p53 pos.	CollXVII pos.	EGFR pos.
Oral cavity n=7	3 (42.9%)	3 (42.9%)	5 (71.4%)	6 (85.7%)	7 (100%)
Oropharynx n=25	15 (60%)	19 (76%)	15 (60%)	16 (64%)	18 (72%)
Hypopharynx n=13	7 (53.8%)	11 (84.6%)	8 (61.5%)	10 (76.9%)	11 (84.6%)
Larynx n=79	38 (48.1)	47 (59.5%)	42 (53.2%)	62 (78.5%)	65 (82.3%)
Total n=124	63 (50.8%)	80 (64.5%)	70 (56.5%)	94 (75.8%)	101 (81.5%)
Chi-square test (p value)	0.7287	0.1097	0.7409	0.4589	0.3626

laryngeal (50%) tumors. The ratio of collXVII positivite supraglottic tumors (80%), was similar to those affecting the larynx (77.8%), or the hypopharynx (76.9%) (p= 0.9678). Neither the p53 positive nor the EGFR positive SCCs showed significant regional difference.

Discussion

According to our results, all the five examined immunohistochemical markers were detectable in tumors of oral cavity, pharynx and larynx. We observed the significant elevation of $p16^{ink4}$ and Ki67 expression along the anatomical localization of SCC. Supraglottic as well as tonsillar and tonsillo-lingual SCCs showed high frequency of positive cells for these antigens as opposed to those affecting the oral cavity, oropharynx without tonsils, larynx without supraglottis and hypopharynx which revealed substantially less positive cells. This feature may correlate with the different developmental routes of these anatomical regions.

The main biological function of $p16^{ink4}$ is to regulate the cell cycle by binding to cyclin-dependent kinases (cdk) 4/6 and preventing the formation of an active cyclin D-cdk4/6 complex. The initial cyclin D-associated phosphorylation of the retinoblastoma protein (Rb), fundamental for cell cycle progression, is therefore inhibited resulting in a G1-arrest [12]. Many early cancers of head and neck show the loss of the chromosomal region which causes the inactivation of $p16^{ink4}$. Studies suggest that loss of $p16^{ink4}$ expression may be associated with poorer prognosis in patients with HNSCC [13, 14].

Ki67 with a unique molecular structure possesses fundamental biological functions that are essential for normal cell proliferation. Since Ki67 protein is present in every dividing cell (G1, S, G2/M phase) but is absent from the resting cells (G0 phase), it is very much suitable for identifying the proliferating fraction of cells. Thus, it provides essential information concerning the replication activity and thus, the potential aggressiveness of a tumor and on the potential response to certain therapy. A high Ki67 labeling index was found to be linked to a reduced time interval until relapse of disease after surgery and adjuvant radiation in head and neck cancer patients [15, 16].

In our study, the high frequency of Ki67 expression is consistent with the rapid growth rate of tonsillar and supraglottic SCCs, but the high expression of $p16^{ink4}$ would rather suggest a better outcome of the disease. Since the cell cycle control machinery can be disrupted at several points, this contradiction needs further studies in order to be explained.

Collagen XVII is a transmembrane protein with collagen-like extracellular domains contributing to the anchorage of undifferentiated proliferating basal keratinocytes to the underlying basal lamina. Collagen XVII was widely expressed from very early stage in the majority of tumor cells in SCC of the skin, including the metastases [11].

Collagen XVII has not been analyzed yet in tumors of the head and neck, thus investigation of this topic offers novel data. We found differing Collagen XVII positivity in some anatomical regions, but regarding positivity ratio, we did not find the difference to be statistically significant.

One of the earliest significant tumor suppressor genes identified in head and neck cancer is p53. However, p53 is

Table 4 Ratio of immunopositive head and neck cancers of the tonsillar versus the oral cavity plus the rest of oropharyngeal cancers

Localization ⇓	$p16^{ink4}$ pos.	Ki67 pos.	p53 pos.	Coll XVII pos.	EGFR pos.
Tonsil n=12	9 (75%)	12 (100%)	8 (66.7%)	11 (91.7%)	11 (91.7%)
Oral cavity—other oropharynx n=20	9 (45%)	10 (50%)	12 (60%)	13 (65%)	14 (70%)
Total n=32	18 (56.2%)	22 (68.7%)	20 (62.5%)	24 (75%)	25 (78.1%)
Chi-square test	0.0977	0.0031	0.7061	0.0917	0.1512

Table 5 Ratio of immunopositive head and neck cancers of the supraglottic versus glottic/transglottic and hypopharygeal cancers

Localization ⇓	$p16^{ink4}$ pos.	Ki67 pos.	p53 pos.	Coll XVII pos.	EGFR pos.
Supraglottic n=25	18 (72%)	20 (80%)	14 (56%)	20 (80%)	24 (96%)
Other larynx n=54	20 (37%)	27 (50%)	28 (51.9%)	42 (77.8%)	41 (75.9%)
Hypopharynx n=13	7 (53.8%)	11 (84.6%)	8 (61.5%)	10 (76.9%)	11 (84.6%)
Total n=92	45 (48.9%)	58 (63%)	50 (54.3%)	72 (78.3%)	76 (82.6%)
Chi-square test	0.0142	0.0081	0.8049	0.9678	0.0891

the most commonly mutated tumor suppressor gene in cancers, for example in head and neck cancer [17]. Mutations in p53 have been correlated with poorer survival and poorer response to treatment [18–20].

Expression of EGFR is a normal finding in many tissues, including dermis, gastrointestinal tract, and kidneys. However, dysfunction of this receptor and its associated pathways occurs in most epithelial cancer and in 80% to 90% of head and neck carcinomas specifically [21, 22]. From a prognostic standpoint, EGFR over-expression and amplification have been demonstrated in retrospective studies to be reliable marker for poor prognosis [23]. Furthermore a specific EGFR tyrosine kinase domain mutation may be a marker for response to chemotherapy [24, 25].

We also detected p53 and EGFR positivity at each anatomical site, but with respect to positivity ratio, no significant difference was observed regarding localization.

Based on the differential expression of Ki67 and $p16^{ink4}$ in head and neck SCC, we suggest that tonsillar (palatine tonsil and lingual tonsil) and other oropharyngeal regions (soft palate, posterior pharyngeal wall)—including tumors of the nearby oral cavity—should be considered separately. It must be further investigated if the distinct biomarker expression correlates with diverse clinical behavior in these groups.

$P16^{ink4}$ positivity in tonsillar tumors is remarkably higher than that of the oral cavity and other oropharyngeal cancer (p=0.0977), but the difference is not significant. In contrast, Ki67 positivity was significantly higher in tonsillar tumors than in tumors of oral cavity and other oropharynx (p=0.0031).

Another statement of our study is that the biological behaviour of laryngeal cancers is different regarding the subregions. It is known from the clinical practice that the three laryngeal levels (supraglottic, glottic, subglottic) show different behaviors from the aspect of local spread and metastases. The spreading pattern of tumors within the larynx is affected by ligaments, connective tissue membranes, and major cartilages of the larynx that inhibits spread of tumors, as well as by soft tissue spaces within the larynx that act as pathways for the spread of tumors within and outside of the larynx [26]. The lymphatic drainage of the three regions of the larynx differs. This feature of tumor behavior may also be explained by the embryologic development of the larynx. The supraglottic larynx is derived from the buccopharyngeal primordium, which develops from the third to fourth branchial arches. The glottis and subglottis, however, are derived from the tracheobronchial primordium from the sixth branchial arch, and are formed by the union of lateral furrows that develop at each side of the tracheobronchial primordium. The difference in tumor behavior is also characterized by gene expression, i.e. we found that $p16^{ink4}$ positivity ratio of supraglottic tumors was significantly higher than that of other laryngeal and hypopharyngeal tumors (p=0.0142). Ki67 positivity of supraglottic tumors was also significantly higher than that of other laryngeal and hypopharyngeal tumors (p=0.0081).

We found a notable similarity between $p16^{ink4}$ and Ki67 positivity in tumors of the tonsils and the supraglottic region (tonsils: $p16^{ink4}$ positivity 75%, Ki67 positivity 100%, supraglottic: $p16^{ink4}$ positivity 72%, Ki67 positivity: 80%).

The similar embryological origin of the lingual tonsil and the supraglottis can be a possible explanation for this phenomenon. The diverse roles played by Ki67 and $p16^{ink4}$ in the regulation of the cell cycle in normal cells may not affect this statement. Our investigations regarding the potential clinical significance of these findings in head and neck cancers by considering the histological grade, TNM stage, overall and disease free survival in different tumor localizations are under way.

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