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RESEARCH

The Role of Protein p16^{INK4a} in Glottic Laryngeal Squamous Cell Carcinoma

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Abstract Head and neck squamous cell cancer (HNSCC) includes tumors of various anatomical sites sharing the common etiological factors. However some differences in pathogenesis and prognosis of HNSCC have been hitherto documented. Laryngeal squamous cell carcinoma (LSCC) is one the most common type of the head and neck cancer. The majority of laryngeal cancers are located in the glottic area. P16 was recently documented to be important prognostic marker in many tumors including HNSCC. The aim of our study was to assess the significance of p16 expression in glottic LSCC. Fifty eight patients after surgical treatment of the glottic LSCC were enrolled in the retrospective study. The p16 expression was immunohistochemically detected and semiquantitatively evaluated in tumor tissue. The results were statistically correlated with clinical and pathological parameters. Protein p16 was expressed in glottic LSCC of 15 patients (25.9 %). Statistically significant higher p16 overexpression was proven in non-smokers in comparison with smokers (75 % versus 18 %; p =0.003). Recurrent cancer was diagnosed in 8 patients (13.8 %), and all these tumors were p16 negative. Our study shows, that p16 expression in glottic LSCC especially in subgroup of non-smokers might be promising prognosticator of better clinical outcome in routine practice. The p16 status did not statistically correlate with cervical lymph node metastases or with grading and staging of cancers, respectively. The preliminary results suggest that p16 overexpression in glottic LSCC may identify patients at low risk of disease recurrence. However, the pathobiology of this tumor as well as predictive role of p16 expression in laryngeal cancer still remains to be better elucidated.

Keywords Laryngeal cancer · Squamous cell carcinoma · p16 protein · Prognostic marker

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous group of neoplasms that represent a clinically challenging disease with more than 551,000 new cases and over 306,000 deaths recorded worldwide in 2008 [1]. Laryngeal squamous cell carcinoma (LSCC), forming more than 95 % of all laryngeal malignant tumors, is one the most common type of the head and neck cancer. The majority of LSCCs are located in the glottic area [1, 2]. Although the principal risk factors for head and neck cancers remain tobacco smoking and alcohol abuse, human papillomavirus (HPV) infection has recently been found to be etiologically associated with 15–35 % of cases of HNSCCs [3]. The etiologic role of "high-risk" human papillomavirus (HPV; in particular type 16 and 18) has been described in HNSCCs, mainly in oropharyngeal cancer [4].

Recently, it was demonstrated that viral infection is a plausible cause for laryngeal carcinoma [5]. However, the causal link between HPV and laryngeal carcinoma remains controversial. The link between HPV infection and development of laryngeal carcinoma is influenced by geographical

factors as it was confirmed in two large case–control studies [6]. The literature data suggest that overexpression of the tumor suppressor protein $p16^{INK4a}$ (hereafter denoted as p16) is strongly related to the active replication of "high-risk" HPV [7, 8].

The prognosis of HNSCC is depended on many tumors and host factors, such as TNM staging and pathological grading or patient performance status, however none of these factors are able to predict reliably the outcome of the disease [9]. Therefore disclosure of the new prognostic and/or predictive markers of HNSCC seems to be lately a big task. Rapidly developing technology in the field of molecular biology enables estimation of control mechanisms of cancer cells at molecular level. Much interest has focused on the proteins participating in the cell cycle control. Defects in the system controlling the cell cycle play an important role in the pathogenesis of cancer, resulting in an increased proliferation of cancer cells [10]. Squamous tumorigenesis is thought to result from successive accumulation of molecular genetic alterations in the squamous epithelium lining the upper aerodigestive tract. Promising tumor marker in this respect seems to be p16 protein having the second most frequently altered expression in tumors after p53. Function of p16 protein consists in negative regulation of retinoblastoma (Rb) protein thus the cell cycle is inhibited and apoptosis is attenuated [7]. Infection by epitheliotrophic HPV was documented to be related to an altered expression of p16. Protein p16 is a member of the INK4 inhibitor kinase family that maps to the 9p21 region, which is frequently deleted in head and neck squamous cell carcinomas and cell lines [11]. The increased expression of p16 in oropharyngeal carcinomas is relatively easy to detect and has been shown to be an surrogate marker of biologically active HPV infection [12]. Majority studies of laryngeal cancer are focused on prevalence and physical status of HPV in these tumors, but do not differentiate subsite of larynx. Association of the p16 overexpression with glottic LSCC and pathobiological role of this tumor marker irrespective to HPV status has not been so far well documented.

Patients and Methods

Fifty eight patients (4 women and 54 men) with the glottic LSCC were enrolled in the retrospective study. All patients had undergone surgical resection of these laryngeal tumors at The Department of Otorhinolaryngology and Head and Neck Surgery, University Hospital in Hradec Kralove, Czech Republic during the years 2001–2009. Five patients with clinically positive lymph nodes underwent a neck dissection. Surgery was followed with radiotherapy in 12 patients. The clinical information, including sex, age, history of smoking, histological grading, pathological and clinical staging (TNM classification [13]) and oncological outcome, were obtained retrospectively from clinical records.

Paraffin-embedded blocks of all studied carcinomas were retrieved from archive file of The Fingerland Department of Pathology, University Hospital, Hradec Kralove, Czech Republic. Tissue sections (4 μm thick) were cut, deparaffinize in xylene, rehydrated in alcohols, and washed twice with water.

One section of each tumor was stained with hematoxylin-eosin and served for confirmation of diagnosis, one section was used for immunohistochemical analysis. Immunohistochemistry assay the CINtec® Histology Kit (Roche mtm laboratories AG, Heidelberg, Germany) was used for the detection of the protein $p16^{INK4a}$. Majority reagents indispensable for immunohistochemical procedure are included in the CINtec® Histology Kit, namely peroxidase blocking reagent, epitope retrieval solution, primary mouse monoclonal antibody against human p16 (clone E6H4), visualization reagent (dextran polymer conjugated with horseradish peroxidase and goat anti-mouse immunoglobulins), and diaminobenzidine (DAB) as chromogene. Samples of cervical intraepithelial neoplasia grade III were used as external positive tissue control for each run. Control slides provided by the manufacturer served as negative controls. Immunostaining was performed on a Ventana Benchmark LT automated immunostainer (Ventana Medical Systems, Inc., TusconAZ). Slides were counterstained with Gill's hematoxylin. Brown staining of tumor cell nuclei and/or cytoplasm was interpreted as positive. The p16 immunostaining was scored according to criteria set out in our previous studies [14, 15] as follows: (0) absent, (+) 1–5 % tumor cells stained, (++) 6–20 % tumor cells stained, (+++) 21–50 % tumor cells stained, and (++++) 51–100 % tumor cells stained. The staining intensity was interpreted as weak, moderate, or strong. For statistical purposes tumors with any p16 expression were consider to be positive irrespective of intensity of staining.

The correlation between the clinicopathological parameters and the p16 expressions was evaluated using the Fisher exact test and Pearson Chi-square test. A P values less than 0.05 were considered to be statistically significant in all statistical analyses. The statistical analysis was performed using SYST AT, version 8.0.

Results

The median age of the patients was 63 years (range 28 to 78 years). Histological diagnosis of laryngeal squamous cell carcinoma as well as histological grading in all 58 cases was established on hematoxylin-eosin stained sections. The histological grading (G1–G3) of tumors was evaluated as follows: G1: 11 carcinomas (19 %), G2: 36 carcinomas (62 %), G3: 11 carcinomas (19 %). According to TNM classification system cancers were scored as follows: pT1a: 37 cases (63.8 %), pT1b: 6 cases (10.3 %), pT2: 12 cases (20.7 %),

pT3: 2 cases (3.5 %), T4a: 1 case (1.7 %). The majority of patients (53/58, 91.4 %) were in the cN0 stage (clinically negative neck node tumors). Patients with clinically positive neck nodes underwent a neck dissection, which in 3 cases confirmed lymph node positivity. None of the patients presented with distant metastases (cM0). Treatment decision-making was based on clinical status of patients and on grading and staging of tumors.

The 3-year disease free survival was 86.2 (50/58) and 13.8 % (8/58) patients experienced with local recurrence. Glottic LSCC of one patient was preceded with the recurrent papillomatosis of larynx for 5 years before diagnosis of cancer. Clinicopathological characteristics are summarized in Table 1.

Protein p16 expression was assessed by immunohistochemistry. The reaction product was evaluated in the individual cancer cells according to nuclear and cytoplasmic dark brown staining. P16 positive cells were easily distinguished from the negative ones. For analysis cases were divided binarily into positive (any p16 positivity, score 1+ to 4+) and negative groups. A total of 15 glottic LSCCs in this study were positive (25.9 %). The majority of p16 expressing tumors showed less than 51 % positive cells. P16 scoring in positive cases is shown in Table 2. The staining intensity was usually interpreted as strong irrespective of staining scores (Table 3). Distribution of p16 positive cancer cell population was patchy ranging from scattered to almost confluent staining pattern (Fig. 1). Statistically significant differences in p16 positivity were found between non-smokers and smokers (75 % versus 18 % of p16 positive tumors; p =0.003). LSCC in group of non-smokers showed predominantly higher p16 score (4+). Recurrent cancer was diagnosed in eight patients (13.8 %), and all these tumors were p16 negative. Correlation between p16 expression and recurrence of tumor was not statistically significant (p =0.097). Cervical lymph node metastases were detected in 3 patients with glottic LSCC (5.2 %), two of them were p16 negative. P16 expression did not statistically correlate with cervical lymph node metastases or with grading and staging of cancers, respectively (Table 1).

Discussion

Head and neck squamous cell cancer includes tumors of the epithelial origin located in oral cavity, pharynx and larynx. Although cancers in these anatomic regions share common risk factors and are often studied together as a single disease, some differences in their pathogenesis and prognosis have been hitherto documented [16]. Therefore studies of site-specific HNSCC are desirable to better elucidate the pathobiology of these tumors. Above mentioned reasons provoked us to the study of the LSCC that is one of the most common head and neck tumors.

Historically, the major risk factors for HNSCC were tobacco and alcohol abuse. Approximately 5 % of all laryngeal carcinomas occur in non-smokers and non-drinkers, suggesting that other etiological factors may be etiologically involved [17]. HPV has emerged as a distinct risk factor for oropharyngeal squamous cell carcinoma, differing from classic tobacco/alcohol-associated HNSCC. HPV as potential etiological factor has been for the first time linked to laryngeal carcinomas more than 20 years ago [17]. Many controversies exist in respect to HPV role in cancerogenesis [5]. The upper aerodigestive tract represents the classical location of HPV infection usually related to laryngeal papillomatosis or uncommon verruca vulgaris caused by “low-risk” HPV (types 6 and 11). Moreover, latent HPV infection was identified in normal laryngeal mucosa approximately in 19 % population [18, 19]. Recent research indicates that “high-risk” HPV (namely types 16 and 18) may play a significant role in the transformation of benign laryngeal lesions as well [5]. This claim could be supported by one of our p16 positive glottic LSCC that arose out of the preceding recurrent papillomatosis. Hobbs et al. in their meta-analysis based on studies of HPV 16 overexpression in HNSCC supported the strongest association between HPV and cancer in tonsils and oropharynx whereas in oral and laryngeal location this link was weaker [20]. Etiologic relationship between HPV and a majority of oropharyngeal squamous cell carcinomas has been established [7, 8, 21, 22]. In oropharynx transcriptionally active HPV is reflected by p16 overexpression (similarly such link was observed in HPV-related cervical carcinomas), and by p21 as well [22–25]. P16 positivity seems to be the indicators of better overall survival and quality of life of patients with oropharyngeal cancer [8, 26]. In contrast to oropharyngeal carcinoma there was not found strong association between expression of p16 and transcriptionally active HPV in the non-oropharyngeal HNSCC. All these conflicting data on the HPV etiology and pathogenesis of laryngeal squamous cell carcinoma initiated the additional studies. Generally, the occurrence of HPV DNA is ranging from 5 to 60 % in LSCC [22]. Many authors point out the considerable influence of HPV infection (subtypes 16 and 18) on carcinogenesis in laryngeal carcinomas with established prevalence about 25 % [5, 27].

Recently has been documented the correlation between the transcriptionally active high-risk HPV infection and p16 overexpression in both oropharyngeal, and laryngeal cancers [7, 15, 17]. According to many authors HPV positive HNSCC present better clinical outcomes compared to those with HPV-negative tumors [28, 29]. The explanation of the above mentioned link between HPV and p16 is based on well-known pathobiological mechanism of transcriptionally active HPV. Human papillomavirus DNA integrated into the genome of the host mucosal epithelial cell initiates overexpression of E6 and E7 oncoproteins. Both proteins enhance p53 degradation,

Table 1 Correlation of p16 positivity with clinical and pathological status

–		p16				P-value*
		Positive		Negative		
–	Number	number	Percent	number	Percent	
No. of patients	58	15	25.9	43	74.1	–
Sex						
male	54	13	24.1	41	75.9	0.563[a]
female	4	2	50	2	50	
Age						
≤60	25	8	32	17	68	0.381[a]
>60	33	7	21.1	26	78.9	
Abuses						
smoker	50	9	18	41	82	0.003[a]
non-smoker	8	6	75	2	25	
TNM staging						
I	43	11	25.6	32	74.4	1.000[a]
II–IV	15	4	26.7	11	73.3	
Histological grading						
G1	11	3	27.3	8	72.7	0.811[b]
G2	36	10	27.8	26	72.2	
G3	11	2	22.2	9	77.8	
Nodal metastasis						
positive	3	1	33.3	2	66.7	1.000[a]
negative	55	14	25.5	41	74.5	
Local recurrence (≤3 year)						
yes	8	0	0	8	100	0.097[a]
no	50	15	30	35	70	

[a] Fisher exact test

[b] Pearson Chi-square test

* Diferrence is significant at the significance level $p < 0.05$

causing a block in apoptosis. E7 protein binds to Rb protein, disrupting E2F/Rb complex. Consequently Rb protein is degraded and cell cycle restriction is removed. Final effect of release a transcription factor E2F is induction of p16 expression [30]. However, despite of lack of detectable HPV DNA in some cases of laryngeal/oropharyngeal squamous cell carcinomas p16 overexpression was revealed and associated with favorable clinical outcome and better response to therapy [21, 31–34]. Traditional prognosticators of aggressiveness and poorer survival outcomes, such as extracapsular spread from cervical nodes, were not found to be significant in p16 positive oropharynx cancer [34, 35].

The role of p16 tumor supressor protein in pathogenesis and in clinical outcome of laryngeal cancer regardless of HPV status still remains to be more elucidated [36]. Anyway p16 protein is intimately linked cell-cycle regulatory system including proliferation and differentiation, senescence, and apoptosis. Alteration of p16 expression is considered to be a common event in many cancers [37]. P16 protein as an inhibitor of cyclin-dependent kinase (CDK) negatively regulates the cell cycle by inactivating CDKs that phosphorylate the retinoblastoma protein (pRb). Pathways of regulatory proteins p15, p21, and telomerase are influenced by possible interaction with p16 [38]. Downregulation of p16 results in abnormally increased tumor proliferation while p16 upregulation inhibites of cyclin-dependent kinases (CDK4andCDK6) to arrest cells in the G1-phase [30]. Therefore the expression of p16 was documented as

Table 2 Review of p16 staining score in positive cases

Score	+	++	+++	++++
Number of tumors	3	3	4	5

+=1–5 % tumor cells stained, ++=6–20 % tumor cells stained, +++=21–50 % tumor cells stained, ++++=51–100 % tumor cells stained

Table 3 Number of p16 positive glottic LSCCs by immunohistochemical staining score and staining intensity

Intensity Score	Weak	Moderate	Strong
+	n=1	n=0	n=2
++	n=1	n=0	n=2
+++	n=0	n=0	n=4
++++	n=0	n=0	n=5

+=1–5 % tumor cells stained, ++=6–20 % tumor cells stained, +++=21–50 % tumor cells stained, ++++=51–100 % tumor cells, n = number of cases

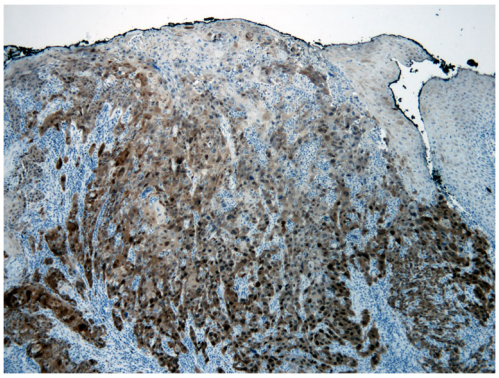

Fig. 1 Glottic LSCC with nuclear and cytoplasmic p16 expression (original magnification 100x)

an important predictor of biological behavior in some malignant tumors, such as melanomas, pancreatic cancer, lung, bladder, and cervical carcinomas [39–43]. Immunohistochemistry was proven to be the best and cost-effective method for p16 detection in tumor tissue [8, 15, 22].

P16 protein is encoded by tumor suppressor CDKN2A gene mapped on the short arm of the human chromosome nine, in the region 9p21. Genetic and/or epigenetic alterations may be influenced by several environmental agents, including tobacco smoke, alcohol abuse, and viruses. Role of smoking in p16 gene inactivation was recently documented in oral and oropharyngeal squamous cell carcinoma [14, 44]. The CDKN2A gene is inactivated in approximately 70 % of human cancers [45]. The main pathways responsible for an inactivation of p16 are loss of heterozygosity (LOH) at chromosome 9p21, methylation of the p16 gene promotor, and point mutation of the gene. The first two events were proven to be the most common in HNSCC [46–50]. Methylation was documented in about 20 % of head and neck cancers and LOH of 9p21 chromosome region was found in up to 72 % of pharyngeal and laryngeal carcinomas [46, 47]. But on the other hand promotor methylation was as frequent as LOH of CDKN2A gene in one study of head and neck cancers [48]. Inactivation of the tumor supressor gene CDKN2A was found as an early event in multi-step process in some HNSCC, namely in oral cancerogenesis [30, 45].

Molecular basis for p16 overexpression in HPV negative cases remains still unclear. P16 upregulation could be probably related to alteration of Rb gene (and thus Rb-p16 pathway) or to innate increasing of p16 expression [21].

Head and neck cancers have been analyzed together without of distinguishing distinct anatomical sites in the majority studies [7, 8, 12, 20, 28, 46, 48, 49, 51]. Behavior of such site – specific HNSCC can differ significantly, suggesting miscellaneous pathways of cancerogenesis and different intrinsic tumor properties. Because of missing the detailed pathobiological and prognostical data in non-oropharyngeal HNSCCs, our study was focused on the glottic laryngeal cancer particularly with the respect to the role of p16 in pathobiology and behavior of this tumor.

P16 positive LSCCs were reported in literature in range from 1 to 58 %. In our study, p16 overexpression was found in 25.9 % (15/58) glottic LSCCs. Given that p16 protein appears to be a suitable marker for detection directly in the tumor tissue, our analysis of p16 prevalence in glottic LSCC is comparable with results of the other studies [8, 15, 17]. Similarly, Tamas et al. [12], Fu et al. [52] and Mendelsohn et al. [51] reported p16 positivity in 39.5 (15/38), 40 (16/40), and 5.6 % (1/18) glottic LSCC, respectively.

P16 overexpression was proven by some authors to be associated with better prognosis of LSCC, while p16 negativity determined tumor progression and poor outcome [16, 38]. Nevertheless, the other studies on the prognostic role of p16 in laryngeal cancer present contradictory results. Duray et al. [53] despite of reported the lowest rate of local recurrence of LSCC with p16 overexpression (20 % recurrence 5 years after diagnosis) did not found no statistically significant differences in p16 expression between the recurrent and disease-free survival cases in their study. Koscielny et al. [46] verified significant association between p16 gene inactivation and poor prognosis of head and neck cancer. Even though in our study the recurrence was revealed in 8 (13.8 %) tumors approximately 3 years after diagnosis and all cases were p16-negative, no statistically significant correlation was found (p =0.097). In agreement with another studies in our cohort of patients the p16 tumor positivity significantly correlated with non-smoker patients whereas in tobacco-related cancers p16 was often absent [16].

Conclusions

Our study shows that p16 overexpression in glottic LSCC significantly correlates tobacco-non-related tumors and suggests to be the early event in pathobiology of these tumors. The expecting prognostic role of p16 in laryngeal cancer has not been proven. The p16 status statistically correlates with neither cervical lymph node metastases, nor grading and staging of cancers. The preliminary results promise that p16 overexpression in glottic LSCC could identify patients at low risk of disease recurrence. However, the pathobiological as well as predictive role of p16 in laryngeal cancer still requires to be better elucidated. Disclosure of new robust surrogate tumor markers of LSCC remains an interesting area of ongoing research.

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