Original paper

Assessment of HPV16 infection in patients with laryngeal cancer

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The purpose of the study was to investigate HPV16 infection in laryngeal cancer patients treated with surgery and adjuvant radiotherapy as well as to analyze treatment results in relation to HPV16 infection and selected clinical, histopathological, and radiotherapy parameters.

A retrospective analysis was performed in a group of 60 patients with squamous cell carcinoma of the larynx treated surgically and qualified for adjuvant radiotherapy at the Oncology Center in Cracow between 1995 and 2001. The studied group consisted of 57 men (95%) and 3 women (5%) of mean age of 56 years. In 13 patients (22%) underweight was noted. In the analyzed material, locally advanced laryngeal cancer prevailed (pT3-pT4) – 52 cases (87%), with the involvement of cervical lymph nodes (pN+) – 32 cases (53%). Histopathological examination revealed that microscopic radicality was not obtained in 18 patients (30%). Human papillomavirus 16 infection status as well as infection type (integrated, episomal, or mixed) were assessed in each patient by means of quantitative polymerase chain reaction (qPCR) using real-time detection.

The 5-year OS, DFS, and LC rates were 45%, 61%, and 69%, respectively. Multivariate analysis revealed that local relapse risk and local failure risk were statistically significantly influenced by underweight and positive surgical margin. Underweight had also a statistically significant impact on death risk. The HPV16 infection was noticed in 4 cancers (6.8%). In all cases it was the same episomal type.

On the basis of our observations it can be assumed that HPV infection does not play an important role in etiology of laryngeal cancer. Although, further study is needed in larger patient populations; optimal methodology for detecting HPV infection should also be determined. Positive surgical margin has a significant effect on worse treatment outcomes. Underweight before radiotherapy diminishes the probability of treatment success and survival of laryngeal cancer patients.

Key words: laryngeal cancer, HPV infection, surgery, radiotherapy, underweight, microscopic radicality.

Introduction

Laryngeal cancer is the most common malignant neoplasm of the head and neck region; in Poland, 2526 new cases were diagnosed and 1610 deaths were recorded in 2015 [1]. The main treatment options for advanced cancer of the larynx are surgery, radiotherapy, and chemotherapy, most often used in combination [2]. Therapeutic management may differ across oncology centers, depending on the centers' own experience and therapeutic guidelines adopted. Treatment decisions in each patient should be made individually and take into account both cancer parameters (localization in the larynx, tumor stage [T], regional lymph node metastases [N], histological type and grade) and patient's parameters (age, performance status, and comorbidities). Patient preferences concerning voice preservation are also of importance. Most indications for adjuvant radiotherapy are based on information obtained from the operative reports and postoperative pathology reports [3, 4]. There are many publications informing on correlations between different clinicopathological parameters and relapse risk of laryngeal cancer [3, 4, 5, 6].

In over 90% of patients, tobacco smoking and alcohol abuse, especially high-proof alcohol, play the leading role in etiology of squamous cell carcinoma of the larynx [7]. In 1982, Syrjanen *et al.* were the first to show the effect of the Human Papillomavirus (HPV) infection on laryngeal cancer incidence [8]. Since then numerous studies have been conducted concerning HPV infection in head and neck cancer patients. It was demonstrated that HPV-positive oral cavity and oropharyngeal cancer patients have better prognosis compared to HPV-negative patients [9, 10]. For laryngeal cancer, reports on the effect of HPV infection on carcinogenesis and treatment outcomes are equivocal [11, 12, 13].

The purpose of the study was to investigate HPV16 infection (its presence and type) in laryngeal cancer patients treated with surgery and adjuvant radiotherapy as well as to analyze treatment results in relation to HPV16 infection and selected clinical, histopathological, and radiotherapy parameters.

Material and methods

Patients

A retrospective analysis was performed in a group of 60 patients with squamous cell carcinoma of the larynx treated surgically in various head and neck surgery departments in southern Poland and qualified for adjuvant radiotherapy at the Oncology Center in Cracow between 1995 and 2001. The study was approved by the Ethical Committee at the Regional Medical Chamber in Krakow (Poland) on 19 September 2012 (109/KBL/OIL/2012) on 19 September 2012 (109/KBL/OIL/2012). No informed consents from patients were required, because during the study no direct contact with patients and use of personal data were necessary. All samples were anonymized.

Histopathological verification of formalin-fixed and paraffin-embedded cancer specimens

For 60 patients, qualified into the study, formalin-fixed and paraffin-embedded (FFPE) cancer specimens (obtained during surgery or biopsy) from were collected. All tumours were subjected to histopathological reevaluation (using archival hematoxylin and eosin-stained slides), in order to confirm diagnosis and to select blocks containing at least 50% of tumor component for DNA selection

DNA isolation

From FFPE blocks selected by the pathologists, DNA was extracted by ReliaPrep™ FFPE gDNA Miniprep System (Promega, Madison, USA). All details concerning this procedure were described earlier [14]. After extraction, the amount and purity of DNA were evaluated spectrophotometrically (NanoDrop Technologies, Inc. USA). The samples were stored at –20°C until qPCR analysis.

Assessment of HPV16 infection status and type by quantitative polymerase chain reaction

In order to normalize qPCR results, each DNA sample was subjected to qPCR for amplification of a 139-bp fragment of *β-actin* gene using TaqMan® Gene Expression Assay (Thermo Fisher Scientific, Waltham, USA) with a mixture of specific primers and MGB probe. All details about this procedure were given earlier [14].

Assessment of HPV16 infection status and type by qPCR was performed on the basis of DNA isolated from archival FFPE tissue sections. HPV16 presence was detected by qPCR, in which amplification of 81 bp fragment of viral *E6* gene was tested. The sequence of primers and Taqman probe (both synthesized by Thermo Fisher Scientific, Waltham, USA) as well as all other details concerning qPCR mixture and reaction's conditions were described earlier [14].

Type of HPV16 infection (integrated, episomal, mixed) was assessed based on the assumption that integration disrupts viral *E2* gene. *E2* copy number was evaluated by qPCR according to Si *et al.* [15] with primers and probe synthesized by Thermo Fisher Scientific, Waltham, USA, as previously described in detail [16]. Type of HPV16 genome was determined based on Ct*E2*/Ct*E6* ratio (Ct – cycle threshold in qPCR). Viral genome was regarded as integrat-

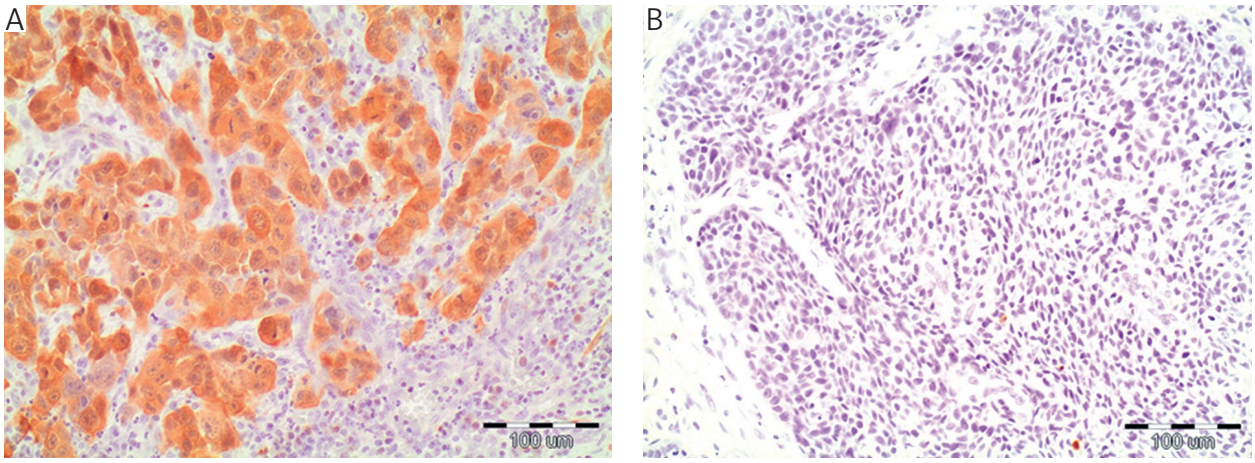

Fig. 1. Experssion of P16 in formalin-fixed paraffin-embedded samples of laryngeal cancers assessed immunohistochemically using CINtec® Histology Kit (Roche, Heidelberg, Germany). A) Tumour with P16 overexpression defined by Lewis *et al.* (2012) as follows: > 75% of positive staining cells or > 50% staining with > 25% confluent positive staining areas (magnification 400×). B) Tumour with lack of P16 overexpression, (magnification 200×)

ed when the Ct*E2*/Ct*E6* ratio equaled 0, as episomal when the ratio was 1 or more, and, finally, as mixed when Ct*E2*/Ct*E6* was between 0 and 1 [15].

P16 immunostaining

P16 expression was evaluated using CINtec® Histology Kit (Roche, Heidelberg, Germany) according to the manufacturer's procedure. All details concernig this procedure were described earlier [10]. Immunopositivity was defined according to Lewis *et al*. [17] as follows: > 75% of positive staining cells or > 50% staining with > 25% confluent areas of positive staining (Fig. 1).

Statistical analysis

The probabilities of 5-year overall survival (OS), disease-free survival (DFS), and local control (LC) were estimated using the Kaplan-Meier method. The log-rank and χ^2 tests were applied to assess statistical differences between groups; the level of statistical significance $p < 0.05$ was adopted. Independent prognostic factors were selected using multivariate Cox analysis.

Results

The studied group consisted of 57 men (95%) and 3 women (5%) of mean age of 56 years (range, 39-71 years). Performance status of most patients was very good or good (ZUBROD 0-1) – 54 patients (90%), while in 6 patients (10%) it was assessed as ZUBROD 2. Detailed data on epidemiological and clinical parameters of the investigated group is summarized in Table I.

In the analyzed cohort, only 2 patients (3%) did not have a history of tobacco smoking; the remaining smoked from 10 to 40 cigarettes per day (mean: 21 cigarettes per day). The mean time of tobacco use was 33 years (range: 10-56 years). Thirty seven patients (62%) reported high-proof alcohol abuse. For men it meant consuming 15 or more drinks per week, whereas for women – 8 drinks.

The mean baseline hemoglobin level was 13.2 g/dl (range: 9.8-15.8 g/dl).

Based on the information from the patients' medical files, nutritional status was assessed using Body mass index (BMI) with the following thresholds adopted: < 18.5 kg/m^2 – underweight, ≥ 18.5 kg m^2 and < 25 kg/m^2 – normal weight, ≥ 25 kg/m^2 – overweight. In 13 patients (22%) underweight was noted, in 8 patients (13%) – overweight, while 39 patients (65%) had normal weight.

In all patients squamous cell carcinoma was diagnosed, including 13 cases (22%) of low-grade (G1) cancer, 35 cases (58%) of intermediate-grade (G2), and, finally, 12 cases (20%) of high-grade (G3) cancer.

Histopathological examination revealed that microscopic radicality was not obtained in 18 patients (30%) (Fig. 2).

On the basis of the pathology reports, postoperative stage of primary tumor (pT) as well as nodal status (pN) were determined. In the analyzed material, locally advanced laryngeal cancer prevailed (pT3-pT4) – 52 cases (87%), with the involvement of cervical lymph nodes (pN+) – 32 cases (53%).

Among 60 patients analyzed, assessment of HPV16 infection status and type was possible for 59 patients; for one patient no DNA was obtained during extraction procedure. In all 59 samples amplification of *β-actin* gene was noted. Mean Ct for *β-ac-*

Table I. Clinical data distribution in laryngeal cancer patients

Parameter/Category	Number of patients n (%)
Sex	
Male	57 (95%)
Female	3 (5%)
Age	
≤ 65 years	48 (80%)
> 65 years	12 (20%)
Performance status (ZUBROD)	
0	2 (3%)
1	52 (87%)
2	6 (10%)
Tumor stage (pT)	
pT2	8 (13%)
pT3-4	52 (87%)
Nodal status (pN)	
pN0	28 (47%)
pN+	32 (53%)
Tumor grade (WHO)	
G1	13 (22%)
G2	35 (58%)
G3	12 (20%)
Microscopically positive margins	
No	42 (70%)
Yes	18 (30%)
HPV16 infection status*	
Negative	55 (93.2%)
Positive	4 (6.8%)
Type of HPV16 infection	
Integrated	0
Episomal	4 (100%)
Mixed	0
P16 overexpression	
Yes	8 (13.3%)
No	52 (86.7%)
Hemoglobin level	
≤ 13.2 g/dl	31 (52%)
> 13.2 g/dl	29 (48%)
BMI (kg/m^2)	
< 18.5	13 (22%)
≥ 18.5 and < 25	39 (65%)
≥ 25	8 (13%)
Cigarettes smoked per day	
0	2 (3%)
≤ 20	47 (79%)
> 20	11 (18%)
Alcohol abuse	
No	23 (38%)
Yes	37 (62%)
Time from surgery to radiotherapy	
≤ 42 days	19 (32%)
> 42 days	41 (68%)
Total dose	
≤ 60 Gy	49 (82%)
> 60 Gy	11 (18%)
Overall time of radiotherapy	
≤ 42 days	33 (55%)
> 42 days	27 (45%)

Table I. Cont.

**Due to unsuccessful DNA extraction, analysis was not possible for 1 sample*
BMI – body mass index

tin was 30.9 ±1.8 (SE). In the group of 59 tumors, HPV16 infection was found in 4 cancers (6.8%). In case of HPV16 positivity, mean Ct values for HPV16 *E6* and *E2* genes were 35.3 ±2.5 and 35.6 ±2.5, respectively. All HPV16-positive tumors showed Ct*E2*/Ct*E6* ratio above 1.0 (mean, 1.01 ±0.0) and the same episomal type of infection. In the group of 60 tumours, P16 overexpression was noticed in 8 (13.3%) samples (Table I, Fig. 1). All P16 overexpressed cancers were HPV16 negative.

Primary treatment method was surgery; the patients underwent total laryngectomy and lymphadenectomy. The patients were treated with conventional radiation therapy using gamma cobalt-60 or 6-MV linac photons. Two lateral opposite fields covering tumor bed and cervical lymph nodes as well as anterior field covering supraclavicluar lymph nodes and tracheostomy site were used. Uninvolved lymph nodes were irradiated to a total dose of 50 Gy in 25 fractions, whereas mean dose to the tumor bed was 61 Gy (median, 60 Gy; range, 60-70 Gy). The spinal cord was shielded after the total dose of 40-44 Gy. Posterior cervical lymph nodes were additionally irradiated with 9-MeV electrons. Mean overall radiation treatment time was 43 days (range, 39-68 days), while mean treatment waiting time from surgery to beginning of radiotherapy was 76 days (range: 26-151 days).

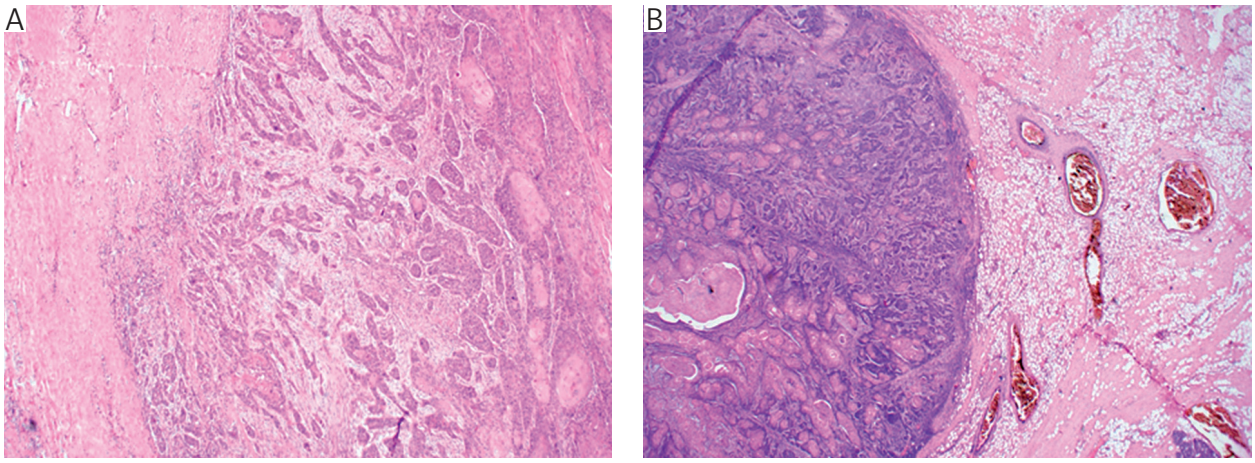

Fig. 2. Two types of laryngeal cancers in hematoxylin and eosin staining of paraffin-embedded samples of laryngeal cancers: A. With cancer infiltration magnification 40×), B. With a well-demarcated "pushing" tumor border (magnification 200×)

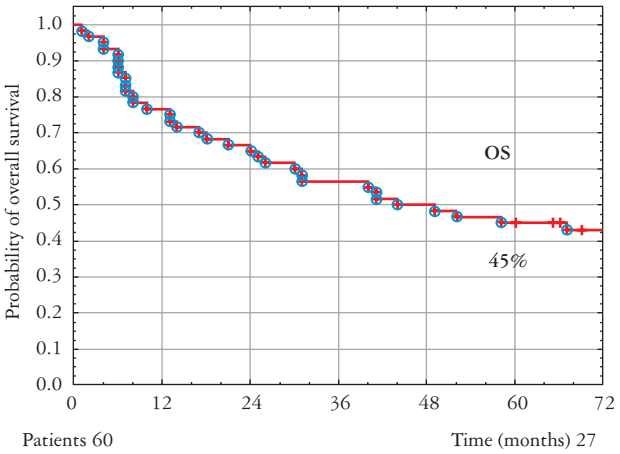

Fig. 3. The probability of 5-year overall survival (OS)

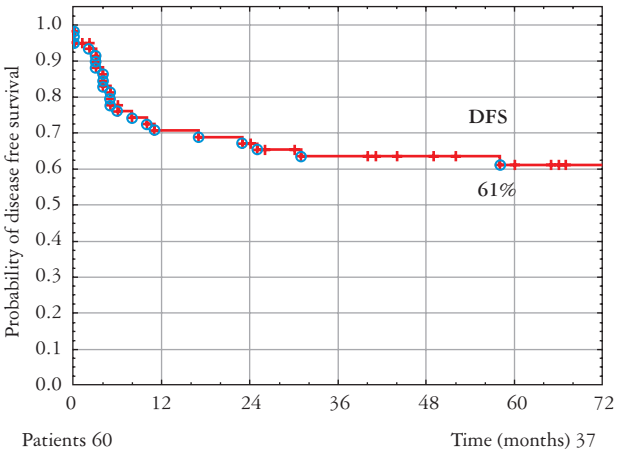

Fig. 4. The probability of 5-year disease-free survival (DFS)

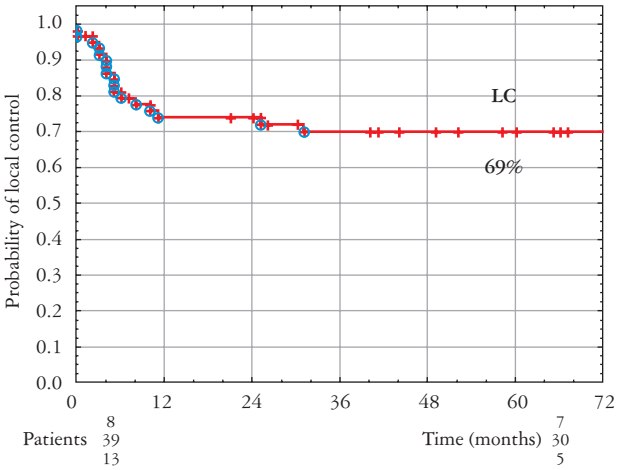

Fig. 5. The probability of 5-year local control (LC)

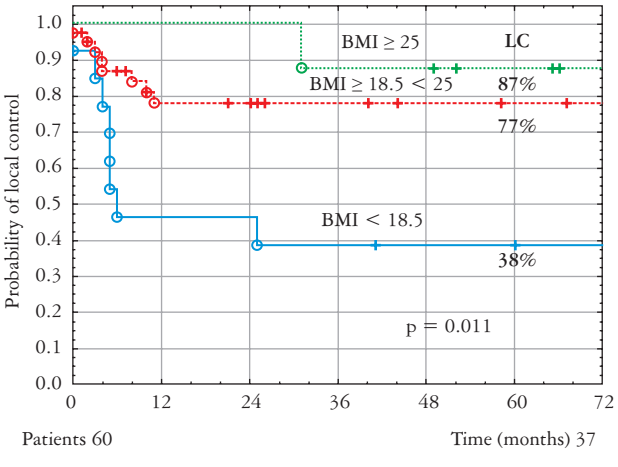

Fig. 6. The probability of 5-year local control (LC) in relation to nutritional status

In the analyzed cohort, mean follow-up period was 73 months (range: 1-235 months). During follow-up 37 patients (62%) died, including 16 patients (27%) of non-oncological causes, 16 patients (27%) of laryngeal cancer, and 5 patients (8%) of another cancer. In the studied group

Table II. The results of univariate analysis of correlations between selected clinical, histopathological, and radiotherapy parameters in relation to 5-year overall survival (OS), disease-free survival (DFS), and local control (LC) rates

Parameter/ Category	5-year LC	5-year DFS	5-year OS
Sex			
Male	68%	59%	45%
Female	100%	100%	33%
	NS	NS	NS
Age			
≤ 65 years	66%	60%	50%
> 65 years	83%	65%	25%
	NS	NS	NS
Performance status (ZUBROD)			
0-1	70%	60%	48%
2	62%	62%	16%
	NS	NS	NS
Tumor stage (pT)			
pT2	88%	88%	88%
pT3-4	66%	55%	37%
	NS	p = 0.043	p = 0.024
Nodal status (pN)			
pN0	72%	68%	57%
pN+	68%	57%	39%
	NS	NS	NS
Tumor grade (WHO)			
G1	76%	60%	53%
G2	63%	58%	42%
G3	80%	72%	41%
	NS	NS	NS
Microscopically positive margins			
No	79%	68%	47%
Yes	48%	43%	38%
	p = 0.024	p = 0.048	NS
HPV16 infection status*			
Negative	83%	68%	42%
Positive	69%	61%	46%
	NS	NS	NS
P16 overexpression			
Yes	88%	87%	75%
No	68%	62%	42%
	NS	NS	p=0.042
Hemoglobin level			
≤ 13.2 g/dl	59%	52%	41%
> 13.2 g/dl	81%	71%	48%
	NS	NS	NS
BMI (kg/m^2)			
< 18.5	38%	38%	23%
≥ 18.5 and < 25	77%	63%	46%
≥ 25	87%	87%	75%
	p = 0.011	p = 0.046	p = 0.021
Cigarettes smoked per day			
0	100%	100%	60%
≤ 20	71%	62%	42%
> 20	63%	54%	63%
	NS	NS	NS
Alcohol abuse			
No	76%	63%	54%
Yes	65%	60%	30%
	NS	NS	NS
Time from surgery to radiotherapy			
≤ 42 days	72%	63%	63%
> 42 days	68%	59%	36%
	NS	NS	NS
Total dose			
≤ 60 Gy	69%	60%	38%
> 60 Gy	71%	63%	72%
	NS	NS	NS
Overall time of radiotherapy			
≤ 42 days	71%	59%	36%
> 42 days	68%	64%	55%
	NS	NS	NS

NS – non significant; BMI – body mass index

of 60 laryngeal cancer patients, 5-year OS, DFS, and LC rates were 45% (Fig. 3), 61% (Fig. 4), and 69% (Fig. 5), respectively.

The results of univariate analysis of correlations between selected clinical, histological, and radiotherapy parameters in relation to 5-year OS, DFS, and LC rates are presented in Table II.

Univariate analysis performed in the investigated group revealed that underweight (BMI < 18.5 kg/m^2) and microscopically positive surgical margin had a statistically significant negative effect on LC and DFS rates. Disease-free survival was also negatively impacted by higher tumor stage (Table II). In the group of underweight patients, 5-year LC rate was 38% compared to 77% in the normal weight group and 87% in the overweight group (Fig. 6), while 5-year DFS was 38%, 63%, and 87%, respec-

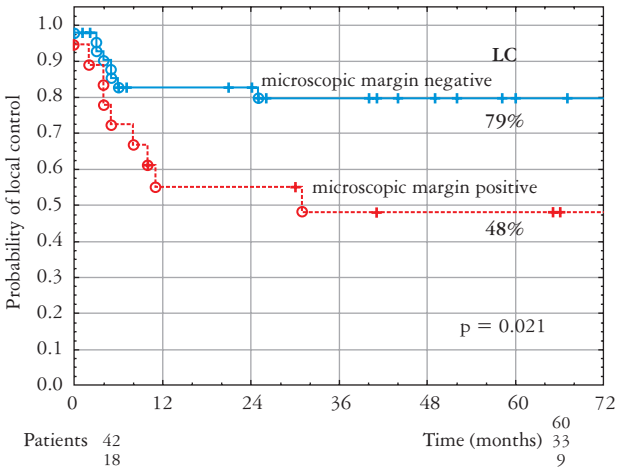

Fig. 7. The probability of 5-year local control (LC) in relation to microscopic surgical margin

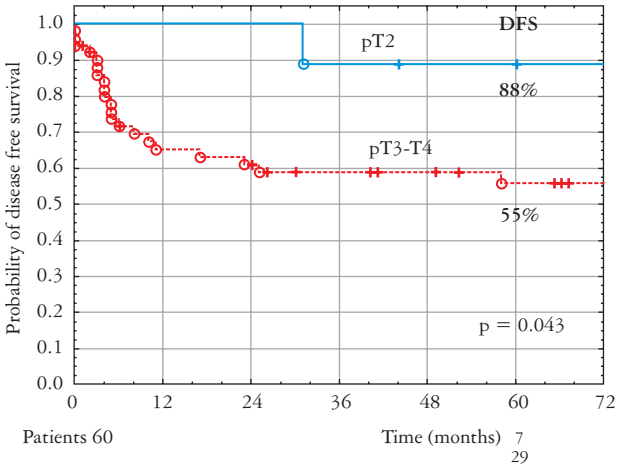

Fig. 8. The probability of 5-year disease-free survival (DFS) in relation to tumor stage

tively. Positive surgical margin was associated with decreasing 5-year LC rate from 79% to 48% (Fig. 7) and DFS rate from 68% to 43%. Five-year DFS decreased with increasing primary tumor stage, from 88% for T2 tumors to 55% for T3-4 tumors (Fig. 8).

Overall survival was significantly negatively affected by lack of P16 overexpression, underweight (BMI < 18.5 kg/m^2) and higher tumor stage (Table II). OS for patients with lack of P16 overexpression was significantly lower (42%) than for those with p16 overexpressed tumours (75%). In the group of underweight patients, 5-year OS was 23% in comparison to 46% in normal weight patients and 75% in overweight patients. Five-year OS in T2 tumors was 88% and decreased to 37% in T3-4 tumors.

Multivariate analysis revealed that local relapse risk and local failure risk were statistically significantly influenced by underweight and positive surgical margin (Tables III and IV). In underweight laryngeal cancer patients, local failure risk was 2.84 times higher than in normal weight patients, whereas cancer-related death risk was 2.11 times higher. Positive surgical margin was related to 3.84 times higher local failure risk and 2.93 higher cancer-related death risk.

In multivariate analysis, underweight had a statistically significant impact on death risk (Table V). Death risk was found to be 2.55 times higher in underweight patients.

Table III. The results of multivariate analysis of relation of selected prognostic parameters and local control

Parameter/ Category	N	Relative risk	p-value
BMI (kg/m^2)			
≥ 18.5	47	1.00	
< 18.5	13	2.84	0.002
Microscopic margin			
Negative	42	1.00	
Positive	18	3.84	0.006

BMI – body mass index

Table IV. The results of multivariate analysis of relation of selected prognostic parameters and disease-free survival

Parameter/ Category	N	Relative risk	p-value
BMI (kg/m^2)			
≥ 18.5	47	1.00	
< 18.5	13	2.11	0.011
Microscopic margin			
Negative	42	1.00	
Positive	18	2.93	0.014

BMI – body mass index

Table V. The results of multivariate analysis of relation of selected prognostic parameters and overall survival

Parameter/ Category	N	Relative risk	p-value
BMI (kg/m^2)			
≥ 18.5	47	1.00	
< 18.5	13	2.55	0.011

BMI – body mass index

Discussion

In the analyzed group of laryngeal cancer patients treated with combined therapy (surgery followed by adjuvant radiotherapy), after a 5-year follow-up period the following rates were observed: LC of 69%, DFS of 61%, and OS of 45%. The obtained outcomes are concordant with relevant literature reporting 5-year LC rates ranging from 63% to 94%

[3, 4, 5, 18]. Literature data shows that radiotherapy increases the effectiveness of combined treatment by 20-30% compared to surgery alone and by 10-40% compared to radiotherapy alone [18, 19, 20, 21].

Although in head and neck cancer patients qualification criteria for postoperative radiotherapy have been well established for many years, when analyzed individually may fail to show prognostic significance. Earlier studies revealed that what affects the outcomes of multimodality treatment is a combination of several risk factors for nodal and/or local relapse [4, 6, 22]. Identification of laryngeal cancer relapse risk groups in surgically treated patients can have a considerable significance for improving treatment results by precise and individual adjustment of physical parameters of radiation therapy (including total dose, radiation technique, irradiated tissue volume as well as treatment time) [4]. Total laryngectomy results in natural voice and breathing path loss. Therefore, it is understandable that efforts are being made to develop vocal organ-preserving treatment strategies. In advanced laryngeal cancer patients radiation therapy results are unsatisfactory [19, 20, 21]. For this reason, numerous attempts have been made to use chemotherapy; they showed that combining chemotherapy with radiotherapy provides vocal organ preservation and further improvement in treatment outcomes of patients with advanced laryngeal cancer [2, 23, 24].

Since the correlation of HPV infection with head and neck cancers has been found, various studies have been conducted showing better prognosis in HPV-positive patients with oral cavity and oropharyngeal cancers [9, 10], which was included in the TNM classification [25]. However, literature data on laryngeal cancer is still inconclusive, therefore, an attempt on detecting HPV infection in the analyzed group of patients was made.

Assessment of HPV16 infection status and type was performed by qPCR on the basis of DNA isolated from archival tissue sections of laryngeal cancer obtained during surgery. HPV16 infection status analysis was possible in 59 cases, in which DNA was obtained during extraction procedure. HPV16 infection was confirmed in 4 laryngeal cancer patients (6.8%). In this group, HPV16 genome type was assessed on the basis of Ct*E2*/Ct*E6* ratio (Ct – cycle threshold in qPCR), which in all patients exceeded 1.0. On this basis, in 4 HPV-positive patients episomal type of infection was recognized. In none of the patients integrated type of HPV infection – relevant in the process of carcinogenesis – was found.

The role of HPV infection in laryngeal cancer has not been unequivocally established so far. According to literature, HPV infection prevalence in squamous cell carcinoma of the larynx varies over a wide range, from 0% [11, 26] to 75% [27, 28]. There are more than 100 HPV types classified in low- and high-risk groups; the most frequently identified type in head and neck cancers is HPV16, while HPV18 is much less common [12, 13, 29, 30]. For this reason, in our material HPV16 was investigated. The discrepancies in the aforementioned results may arise from different HPV detection methods. To the best of our knowledge, in none of the Polish studies on HPV status in patients with laryngeal cancer HPV infection type was investigated (episomal, integrated, mixed). Ours is the first Polish study determining HPV infection type in laryngeal cancer patients; all cases showed episomal type of infection and in none of them integrated type was found. The progression of intraepithelial lesions in the larynx requires viral *E6* and *E7* oncogenes to be expressed and E6 and E7 oncogene proteins produced. Viral oncoproteins are able to bind with cellular products of *p53* and *pRb* suppressor genes, which leads to cell cycle disorder and apoptosis arrest followed by epithelial cell transformation [31]. In the majority of infections with high-risk types of HPV, increased E6 and E7 expression follows integration of the virus with the genome of host cell. During this process, the open reading frame of viral *E7* gene is disrupted, which causes inhibition of expression of this gene and, consequently, uncontrolled expression of viral *E6* and *E7* oncogenes. Therefore, identification of the integrated type of viral genome is based on the assumption that *E2*/*E6* ratio equals zero. On the other hand, episomal type of viral DNA should contain at least the same copy number of *E2* and *E6* with the *E2*/*E6* ratio ≥ 1. Ct*E2*/Ct*E6* ratio ranging from 0.0 to 1.0 indicates the presence of both integrated and episomal viral genome types in the analyzed material (mixed type). It is worth mentioning that there are some controversies about the cut-off value of the Ct*E2*/Ct*E6* ratio adopted for different genome types. In the presented analysis, similarly to other authors [32, 33], the cut-off value for the episomal type was 1.0. However, based on an analysis of solutions containing a series of dilutions of two plasmids (the first one of the episomal type – increasing dilution, the second one of the integrated type – decreasing dilution), Fujii *et al.* have established [34] the cut-off value of the Ct*E2*/Ct*E6* ratio as 0.79. At the same time, Sounier *et al.* [35] recommended adopting the cut-off value of 0.8, because above this value a significant variability of the Ct*E2*/Ct*E6* ratio was observed between the results obtained in subsequent repetitions for the same sample. Meanwhile, studying plasmid constructs of HPV16 DNA of different geographic variants (European, Asian-American, African-1 and African-2), Jiang *et al.* [36] found that for the European variant of the integrated type the Ct*E2*/

C*tE6* ratio was close to 1.0. Consequently, considering other authors results, in the presented analysis the adopted cut-off value was 1.0 [32, 33].

High prevalence of HPV infection in laryngeal cancer observed in certain studies can be overestimated as a consequence of positive HPV DNA testing but inactive viral infection [12]. Earlier reports indicated that only active HPV infection, during which the virus integrates with the host genome, can play a role in the process of carcinogenesis [31]. In a meta-analysis of 12163 head and neck cancer patients, the authors noted active HPV infection in 19.1% of laryngeal cancer cases [13]. However, in a group of 1042 laryngeal cancer patients from 29 countries, Castellsagué *et al.* observed active HPV infection in 1.5% of patients worldwide and in 1.2% of European patients [30].

A wide range of reported HPV infection prevalence in head and neck cancers, including laryngeal cancer, can also result from differences in infection types detected in various geographic regions, tumor location, study size, poor quality of analyzed samples, or sample storage conditions [13, 30]. Survival analysis was not conducted in our study, because the integrated type of HPV was not found in the investigated group. These observations require confirmation in further studies with larger groups of patients. In turn, expression of P16 is known surrogate marker of HPV infection [37] However in the present study we have found that P16 overexpression was found in 8 tumours (13.3%) and none of these tumours were HPV16 positive. These results confirm that P16 analysis is characterized by relative low specificity in detection of HPV infection and may generates false positive results [37]. Overproduction of P16 can be namely caused not only by HPV infection, but also by oncogenes activation, DNA damage or accelerated cellular senescence [38]. Therefore, summarized presented by us results concerning HPV prevalence in laryngeal cancers, it should be state that our resuts suggest that HPV does not play an important role in laryngeal cancer etiology and prognostification.

Multivariate analysis showed that positive surgical margin and BMI before radiotherapy statistically significantly affected treatment outcomes.

In the analyzed group, positive surgical margin was found in 30% of patients. According to the literature, lack of microscopic radicality is observed in 20-70% of cases [3, 4].

Positive surgical margin was related to 3.84 times higher local failure risk and 2.93 times higher cancer death. Numerous studied showed that relapse rate after combined therapy (surgery with adjuvant radiotherapy) was lower by about 30% if negative surgical margin was achieved [3, 4, 21, 39]. This is concordant with our earlier results; positive surgical margin in laryngeal cancer patients treated with surgery followed by radiotherapy was related to statistically significantly decreased 5-year LC rate from 91% to 76%, 5-year DFS rate from 75% to 50%, and 5-year OS rate from 79% to 61% [4]. Hinerman *et al.* also noted decreased 5-year LRC rate from 89% to 56% with positive surgical margin [3]. In a group of 138 T3N0–T4N0 laryngeal cancer patients, Skóra *et al.* reported prognostic significance of surgical margin status; 5-year DFS after microscopically radical surgery was 82% compared to 72% in the case of positive surgical margin [5]. Meanwhile, Johnson *et al.* found that in cases with positive margin combined therapy of surgery and radiation was more effective than surgery alone [40]. In the study by Peters *et al.* a significant predictive value of surgical margin status was not confirmed, however, in patients with positive margin and unfavorable tumor location higher irradiation doses were applied compared to patients with negative margin and different tumor location [6]. Studying a group of 139 patients with cancer of the larynx, Sarantini *et al.* also did not show statistically significant impact of surgical margin [41].

In the analyzed group, underweight (BMI < 18.5 kg/m^2) before radiotherapy was related to 2.84 times higher failure risk, 2.11 times higher cancer death risk, and 2.55 times higher death risk. The results of a previous study including 541 patients with T1 glottic cancer also showed that underweight had statistically significant negative impact on LC, disease-specific survival, and OS. In underweight group, death risk was 1.9 times higher than in normal weight group [42]. For the purpose of the analysis of nutritional status BMI was used in both studies, which is a parameter widely adopted by many researchers. The problem of malnutrition is frequent in head and neck cancer patients. The researchers report that underweight is often related to higher cancer stage at diagnosis and negatively impacts treatment outcomes [43, 44]. In a literature review including 8306 patients with head and neck cancer the authors observed the effect of elevated BMI on higher probability of OS, DFS, and LC compared to normal weight and underweight groups [43]. Another study in a cohort of 473 laryngeal cancer patients showed that BMI significantly affected survival; 5-year OS in overweight, normal weight, and underweight group was 87.2%, 78.0%, and 34.9%, respectively [44]. In a group of 788 laryngeal cancer patients, Riele *et al.* observed a negative impact of underweight on OS. They advocated that BMI can be a predictive factor for OS [45]. It was also confirmed that head and neck cancer patients with weight loss $>10\%$ within 6 months before treatment have a high risk of severe postoperative complications [46].

In conclusion, on the basis of our observations it can be assumed that HPV infection does not play

an important role in etiology of laryngeal cancer. Although, further study is needed in larger patient populations; optimal methodology for detecting HPV infection should also be determined. Positive surgical margin has a significant effect on worse treatment outcomes. Underweight before radiotherapy diminishes the probability of treatment success and survival of laryngeal cancer patients.

The authors declare no conflict of interest.

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