

Aim of the study was to evaluate the potential role of regulatory and proinflammatory cytokines IL-23 and IL-17 as Th17 lymphocyte activity markers in relation to invasiveness in laryngeal cancer.

Material and methods: The immunological analysis was conducted in 50 patients treated for squamous cell laryngeal carcinoma and 30 healthy volunteers as controls. The levels of IL-23 and IL-17 in supernatants of purified peripheral blood mononuclear cell cultures were determined by using the enzyme-linked immunosorbent assay (ELISA). The clinicomorphological criteria included pTNM, stage, G, and the total tumour front grading (TFG) score.

Results: Our data demonstrated higher concentrations of IL-23 in patients as compared to controls ($p = 0.0001$). No statistical difference for IL-17 in these groups was observed. Our study revealed significant dependences in IL-23 expression on pT ($p = 0.04$), histological differentiation ($p = 0.04$), and TFG total score ($p = 0.02$). Advanced tumours (pT3–pT4) with higher grade (G2–G3) and higher invasiveness (> 14 TFG points) were characterised by elevated IL-23 levels in PBMC supernatants. Our data did not indicate a relationship between cytokine levels and three- and five-year survival. However, a tendency towards lower content of IL-23 in PBMC cultures in patients who lived longer than five years after treatment was noted. The relationships between IL-17 level in PBMC cultures and clinicomorphological and prognostic parameters have not been disclosed.

Conclusions: The results of this study suggest the importance of regulatory cytokine IL-23 in determining the aggressive potential of laryngeal carcinomas.

Key words: laryngeal carcinoma, IL-23, IL-17, tumour front grading.

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Expression of Th17 cell population regulatory cytokines in laryngeal carcinoma – Preliminary study

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Introduction

Th17, a population of T helper lymphocytes, producing interleukin (IL)-17, a proinflammatory cytokine that enhances T-cell priming and stimulates the production of other interleukins and molecules such as IL-1 β , IL-6, TNF, NOS-2, as well as chemokines. Th17 cells are in turn produced as a result of naïve CD4+ T-cell stimulation by IL-23, IL-6, and TGF- β 1. The most notable role of IL-17 is its involvement in inducing and mediating proinflammatory responses, e.g. production of many cytokines, chemokines, and prostaglandins, enhancement of dendritic cell maturation, and stimulation of macrophages [1]. Interleukin 23 is a heterodimeric cytokine produced by dendritic cells and macrophages. The IL-23 receptor complex is expressed in a number of cells, including natural killer cells, monocytes, macrophages, dendritic cells, and CD4+ T cells [1]. It appears that the same processes and relationships that are involved in the inflammation may also stimulate cancer growth [2]. Nevertheless, the participation of Th17 cells in anti-tumour immunity remains poorly understood. Cytokines IL-17 and IL-23 have pleiotropic effects. They may be tumour-promoting or tumour-suppressing factors [3–5]. For instance, IL-17 promotes an anti-tumour cytotoxic T-cell response leading to tumour regression. On the other hand, by facilitating angiogenesis and egress of tumour cells from the primary focus, IL-17 promotes tumour growth [6, 7]. It is believed that tumour growth, particularly in the early stages, is triggered by the IL-17 inflammatory effect on the activity of IL-8 and IL-6, and is enhanced by the infiltration of immunocompetent cells to the tumour. While IL-17 can promote the cells leading to tumour regression, IL-23 contributes to the impairment of specific neoplastic cytotoxic CD8+ T lymphocytes and reduces cell infiltration into the transformed tissue [2, 6]. IL-23 may promote tumour progress by activation of neutrophils and macrophages, as well as enhancement of angiogenesis. Moreover, by promoting upregulation of the matrix metalloprotease MMP9, IL-23 acts as a tumour stimulating factor [2]. Numerous data indicate the suppressive effect of cytokines secreted by Th17 cells [8–10]. Assessing the interaction between cancer cells and immune cells, including Th17 cells and secreted IL-17 and IL-23 in relation to tumour aggressiveness, is a leading issue in oncological immunology. Nevertheless, little is known about the possible function of Th17 lymphocytes in the development and progression of laryngeal carcinoma.

The aim of this study was to evaluate the potential role of IL-23 and IL-17, the regulatory cytokines of Th17 cell populations, and to assess the relationships with tumour invasiveness according to clinicomorphological features such as pTNM, G, stage, and tumour front grading score in squamous cell carcinoma.

Material and methods

The study involved a group of 50 patients (48 men, 2 women) with laryngeal squamous cell carcinoma (aged 45–79 years; mean age 62.5 ±8.3 years) and a control group of 30 healthy volunteers (aged 40–65 years; mean age 54.2 ±5.9 years). The criteria for patient participation in this study were as follows: 1) pathologically confirmed diagnosis of carcinoma planoepitheliale; 2) primary surgical resection without receiving prior immuno-, radio-, or chemotherapy; and 3) absence of distant metastasis.

The clinicomorphological characteristics of the studied group are shown in Table 1.

Histological classification and morphological features

Morphological assessment of tumours was performed on archival paraffin-embedded tissue samples. All specimens were estimated according to the criteria conducted in accordance with the AJCC TNM 2010 classification for laryngeal cancer [11]. Morphological analysis was performed on H&E-stained sections from the most invasive, peripheral zones of the cancer infiltration, according to tumour front grading (TFG), a reliable and precise histological method for the evaluation of neoplastic progress [12]. The histological parameters: three tumour-related features (cytoplasmic differentiation, nuclear polymorphism, number of mitoses) and three adjacent stroma-related character-

istics of the peripheral edge of tumour infiltration (mode of invasion, depth of invasion, and plasmalymphocytic infiltration) were assessed in at least five different regions of the peripheral part of the tumour (magnification 200×, number of mitoses magnification 400×). Each factor was graded according to a scale ranging from one to four. The numeric morphological TFG score was computed as a sum of six morphological features, with a maximum score of 24 points. According to the TFG total score and results for laryngeal carcinomas, tumours in this study were divided into three groups: 6–13, 14–17, 18–21 TFG points.

Lymphocyte isolation and ELISA tests for IL-23 and IL-17 measurement

For peripheral blood mononuclear cell (PBMC) isolation the venous blood of each patient was obtained (10 ml) and transferred to test tubes containing heparin (10 U/ml). The control blood samples were obtained from 30 healthy volunteers without a history of malignancies or autoimmune disorders. PBMCs were isolated by Ficoll-Hypaque density gradient and resuspended in RPMI 1649 medium to obtain the concentration of 1×10^6 cells/ml. The recovered cells were checked and counted for viability with Trypan Blue staining method. The isolated PBMC cultures were incubated for 21 hours at 37°C in a humidified atmosphere with 5% CO₂ (Cellstar Incubator) in 96-well plates in a final volume of 0.2 ml (per well). The supernatants of cultures were collected and the secretion pattern of IL-23 and IL-17 was measured with specific enzyme-linked immunosorbent assay ELISA Kit (R&D Systems, Inc.; Minneapolis, MN, USA) according to the manufacturer's instructions. Absorbance was measured with an ELISA reader (Multiscan RC 351). The sensitivity of this assay was 6.8 pg/ml for IL-23 and < 15 pg/ml for IL-17. The investigations were performed with the approval of the Bioethical Commission of the Medical University of Lodz and the National Science Council, Poland (No. RNN/60/13/KE).

Statistical analysis

The statistical analyses were performed using the IBM SPSS STATISTICS 21 (Business Machines Corp., USA) software package. Distributions of quantitative variables were described using means and standard deviations. Since levels of IL-17 and IL-23 expression did not show normal distribution (according to the results of Shapiro-Wilk normality test) the non-parametric statistical tests: Mann-Whitney *U* test and Kruskal-Wallis test with *post hoc* multiple comparisons with Bonferroni correction, were used to identify the relationship between cytokines expression and clinicopathological parameters. A *p*-value of less than 0.05 was considered as statistically significant.

Results

Analysis of IL-23 and IL-17 secretion in cases and control group

Analysis of the data showed a significant difference of the average concentration of IL-23 in the supernatant of PBMCs obtained from patients with squamous cell laryn-

Table 1. Clinicopathological characteristics of laryngeal cancers

	Variable	Cases, n (%)
Surgical treatment	Total laryngectomy	33 (66)
	Partial laryngectomy	17 (34)
	Neck dissection (–)	25 (50)
	Neck dissection (+)	25 (50)
	Selective neck dissection	21 (42)
	Radical neck dissection	4 (8)
Tumour size (pT status)	pT1	6 (12)
	pT2	13 (26)
	pT3	16 (32)
	pT4	15 (30)
Nodal metastases (pN status)	pN0	40 (80)
	pN1–3	10 (20)
Degree of differentiation (grade)	G1	9 (18)
	G2	34 (68)
	G3	7 (14)
TFG total score	6–13 points	19 (38)
	14–17 points	16 (32)
	18–21 points	15 (14)
Survival	< 3 years	8 (16)
	3–5 years	11 (20)
	≥ 5 years	31 (64)

geal carcinoma, as compared to the level of this cytokine in the control group ($p < 0.0001$). The mean concentration of IL-23 in cases was higher in comparison with cytokine secretion in the control group (198.3 ± 303.9 pg/ml vs. 12.6 ± 3.7 pg/ml, for cases and controls, respectively). No significant differences were disclosed between IL-17 mean concentrations in both groups, although slightly higher values of IL-17 concentration were noted in the control group (9.9 ± 24.0 pg/ml vs. 12.9 ± 43.0 pg/ml, for patients and controls, respectively).

The association of IL-23 and IL-17 expression with clinicomorphological features

Subsequently, the pattern of IL-23 and IL-17 expression in purified PBMC cultures from patients treated with laryngeal carcinoma with clinicomorphological parameters was compiled. The present study demonstrated that the increased expression of IL-23 in laryngeal carcinoma cases was an indicator of more advanced neoplastic lesions. Statistical analysis confirmed significant relationship between IL-23 level and local extension of the tumour pT ($p = 0.04$). The presence of the higher content of IL-23 in supernatants of PBMCs was more frequent for tumours with higher pT feature. Moreover, a significant difference between pT2 and pT3 subgroups was disclosed ($p = 0.03$). Similarly, statistical analysis identified a positive association between IL-23 level and histological differentiation ($p = 0.04$) and between the G1 and G3 subgroups ($p = 0.02$). The higher concentration of IL-23 in PBMC cultures was more characteristic for less differentiated tumours. No statistical dependences were noted for pN and stage. However, a clear tendency towards higher values for IL-23 in laryngeal carcinomas characterised by the nodal metastases (pN1–3) and higher clinical advancement (SIII–IV) was noted. Statistical evaluation of the quantitative analysis results and the clinicomorphological features of laryngeal carcinomas showed that the expression of

IL-23 in PBMCs isolated from patients was significantly different depending on the total score of tumour front grading classification ($p = 0.02$). The presence of the higher content of IL-23 in supernatants of PBMCs was more frequent for tumours with more aggressive behaviour determined by higher total score of TFG scale (characterised by 14–21 points) in comparison with less invasive carcinomas (not exceeding 6–13 points in TFG) ($p = 0.02$). Unfortunately, no correlation between the level of IL-17 and the aggressiveness of tumours studied according to both, pTNM classification and TFG scale was confirmed. The IL-23 and IL-17 expression in PBMC cultures in the laryngeal carcinoma group studied and the statistical analysis results are shown in Table 2 and Table 3.

The association of IL-23 and IL-17 expression and three-year and five-year survival

The relationship of IL-23 and IL-17 level in supernatants of purified PBMCs with three-year and five-year survival of patients with laryngeal carcinoma was analysed. Statistical analysis did not disclose significant differentiation of both, IL-23 and IL-17 concentration in relation to patient survival. However, a tendency towards lower content of IL-23 in PBMC cultures in patients who lived longer than five years after treatment as well as higher values for IL-17, for whom the survival period was over three years, was noted. The IL-23 and IL-17 levels in PBMC cultures in patients with laryngeal carcinoma and statistical analysis results are shown in Figs. 1 and 2.

Discussion

In recent decades, numerous studies have confirmed the dependence of immune system activity and both, the development and aggressiveness of cancers of various origins. Nevertheless, little is known about the possible role of IL-17 and IL-23 in the development and progression of laryngeal carcinomas.

Table 2. Associations between clinicopathological characteristics and expression of IL-23

	Variable	IL-23 [pg/ml]	p^a	p^b
Tumour size (pT status)	pT1	174.5 \pm 224.6	0.04	pT2 vs. pT3 $p = 0.03$
	pT2	111.9 \pm 175.7		
	pT3	288.2 \pm 404.2		
	pT4	230.1 \pm 241.8		
Nodal metastases (pN status)	pN0	190.1 \pm 288.2	> 0.05	> 0.05
	pN1-3	235.0 \pm 336.9		
Degree of differentiation (Grade)	G1	86.8 \pm 162.6	0.04	G1 vs. G3 $p = 0.02$
	G2	221.7 \pm 355.6		
	G3	211.9 \pm 272.5		
TFG total score	6–13 points	116.2 \pm 198.3	0.02	6–13 vs. 14–17 $p = 0.02$
	14–17 points	253.4 \pm 379.4		
	18–21 points	230.1 \pm 303.9		
Survival	< 3 years	238.9 \pm 336.9	> 0.05	> 0.05
	3–5 years	221.7 \pm 349.1		
	\geq 5 years	155.4 \pm 242.6		

Results are given as mean \pm standard deviation

^aDifferences between two groups were evaluated with Mann-Whitney U test, differences between three or four groups were evaluated with Kruskal-Wallis

^bMultiple comparison for Kruskal-Wallis test with Bonferroni correction

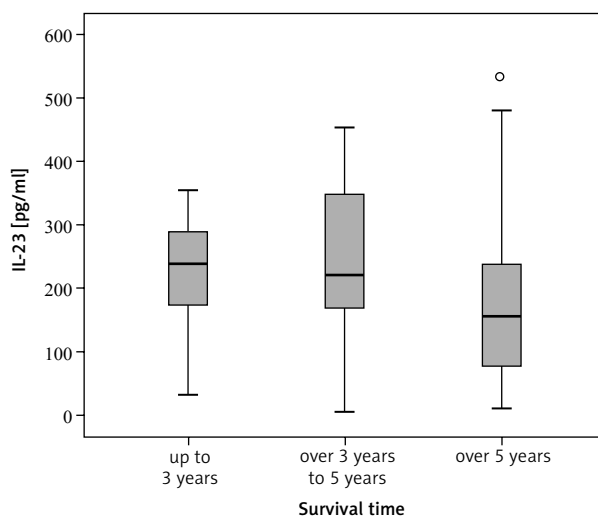
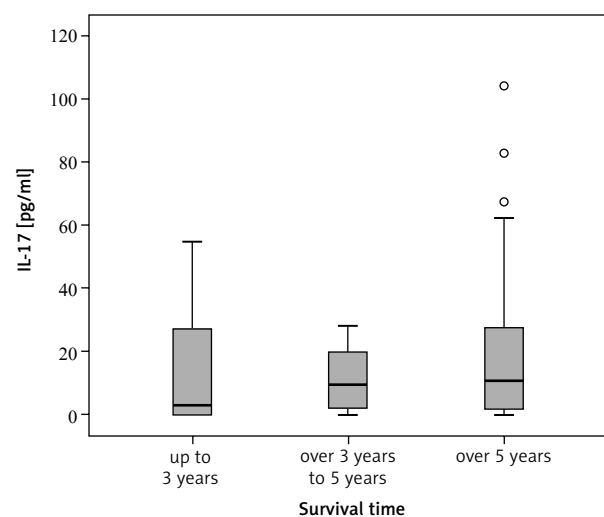
Table 3. Associations between clinicopathological characteristics and expression of IL-17

	Variable	IL-17 [pg/ml]	<i>p</i> ^a	<i>p</i> ^b
Tumour size (pT status)	pT1	5.5 ±24.0	> 0.05	> 0.05
	pT2	12.3 ±32.1		
	pT3	6.7 ±23.7		
	pT4	10.9 ±26.8		
Nodal metastases (pN status)	pN0	10.7 ±31.3	> 0.05	> 0.05
	pN1–3	8.8 ±23.8		
Degree of differentiation (grade)	G1	6.6 ±23.7	> 0.05	> 0.05
	G2	10.6 ±28.4		
	G3	10.9 ±23.8		
TFG total score	6–13 points	10.4 ±24.0	> 0.05	> 0.05
	14–17 points	10.7 ±30.2		
	18–21 points	4.0 ±21.7		
Survival	< 3 years	3.2 ±33.0	> 0.05	> 0.05
	3–5 years	9.9 ±20.1		
	> 5 years	10.9 ±31.3		

Results are given as mean ± standard deviation

^aDifferences between two groups were evaluated with Mann-Whitney U test, differences between three or four groups were evaluated with Kruskal-Wallis

^bMultiple comparison for Kruskal-Wallis test with Bonferroni correction

**Fig. 1.** The concentration of IL-23 in the group studied depending on the 3-year and 5-year survival time**Fig. 2.** The concentration of IL-17 in the group studied depending on the 3-year and 5-year survival time

The findings of our study into IL-23 correlate with the results obtained by other authors who studied various types of tumours, and tumours of other localisation [13–16]. It should be stressed that, despite numerous scientific publications on the role of IL-17 and IL-23 in cancer, only a few published works have dealt with tumours of the head and neck [17–19]. Meng *et al.* [17] assessed the level of IL-17 in tumour-associated macrophages in three groups of patients: with laryngeal squamous cell carcinoma, patients with cancer of adjacent tissues as the control group, and with chronic hypertrophic laryngitis. The expression of the serum level of IL-17 was similar in the three studied groups, while relatively high levels of IL-17 were noted in macrophages in two other assessment methods in patients with LSCC [17]. Kesselring *et al.* [18] found that lymphocytes Th17 and produced IL-17 have a substantial impact on the carcinogenesis of head and neck squamous

cell carcinoma as well as on their metastasis formation. The tissues of tumour and lymph nodes are infiltrated by a huge number of Th17 cells representing an important fraction of tumour-infiltrating lymphocytes (TILs). According to the above authors, proliferation and angiogenesis of HNSCC are impaired in the presence of Th17 cells. Garley *et al.* [19] assessed the levels of IL-17E and IL-17A in patients with oral epithelial squamous cell carcinoma. They showed that the levels of both cytokines were higher both in cell supernatants and in the blood serum of the patients compared to a control group of healthy subjects. However, no correlation was found between the protein expression in the cells or the concentration of supernatants of the patients with different stages of disease.

Many authors confirm that an increase in immunocompetent cell activity may be an indirect index of advancement of the neoplastic process [2, 4, 5, 13, 18]. For instance,

Matowiecka-Karna *et al.* [13] observed increased levels of IL-6 and IL-23 in the blood serum of patients with gastric cancer as compared to healthy subjects. However, lower levels of both cytokines were found in patients with metastatic gastric cancer. Yaghoobi *et al.* [14] noted a substantial rise of IL-23 levels in the anal mucosa of HIV-positive patients with anal cancer as compared to patients without anal cancer. Wu *et al.* [20] observed in patients suffering from early recurrence of primary hepatocellular carcinoma (HCC) higher pre-therapy serum levels of IL-17 than in patients who did not have recurrence after a radical hepatectomy. Zhang *et al.* [23] also found that the main immunological risk factors for the prognosis of non-small cell lung cancer (NSCLC) are negative CD4 expression and positive IL-17 and Foxp3 expressions. However, opposing results were obtained by Hus *et al.* [24]. They showed that higher levels of Th17 and IL-17A were associated with less advanced clinical stage of chronic lymphocyte leukaemia (CLL). Th17 and IL-17A levels were lower in patients with adverse prognostic factors. A number of authors underline the possibility of utilising the research for cancer prevention. Yao *et al.* [25] revealed two genetic variants for IL-23R, rs6682925, and rs10889677, which play a particular role in the pathogenesis of multiple cancers. This was confirmed by Zhou *et al.* [26], who in their studies pointed to the genetic variant rs10889677, which increases the probability of malignant transformation of a great number of solid tumours.

Multidirectional studies presently being conducted are searching for the mechanisms that induce the onset of carcinogenesis and tumour growth. Some authors indicate the role of equilibrium between IL-12 and IL-23 in maintaining cancer cells in a state of immunological quiescence [3, 27–30]. Kortylewski *et al.* [3] showed that IL-12 significantly boosts precancerous immunity through stimulation of lymphocytes Th1, differentiation of cells, and cytotoxic stimulation of lymphocytes T and natural killer cell cytotoxicity. Interleukin 23 had pro-cancerous properties, and the expression of IL-23 was induced by Stat3, which is produced mainly by tumour-associated macrophages. Choi *et al.* [27] in their studies into oncolytic adenoviruses, which inhibit IL-23 expression, pointed out their role in immunotherapy since they may increase antitumour immunity. Cui *et al.* [29] investigated the level of IL-17A in the microenvironment of colorectal cancer. The IL-17 level gradually increased along with the severity of dysplasia. The level of IL-17 was markedly higher in cases of colorectal cancer. Omrane *et al.* [30] noted a significant association between IL-17F and IL-23R polymorphism and the location, histology, and architecture of tumours in patients with colorectal cancer (CRC). They also found a significant relationship between the increased level of IL-17F and an increased risk of TNM advanced stage. A number of authors indicate the necessity of further studies into IL-23 since it seems to be a novel appropriate remedy that could be employed in cancer treatment, and the possibility of monitoring IL-23 levels as a novel therapeutic target, which gives hope for the inhibition of cancer progression [16, 31–33].

The results of this study should be interpreted with caution, and they are difficult to compare with the results

of other authors due to the different localisation and types of cancers, the use of different study methods (ELISA, Western blot, RT-PCR), and the variety of materials (serum, tissues, cell lines, and laboratory animals). Moreover, our study material included a relatively small group of patients with laryngeal carcinoma. Additional research to confirm these findings in a larger group and further studies with regard to relations with other immunological parameters that affect the function of immunocompetent cells, including Th17 lymphocytes, are mandatory [34]. Regardless of these limitations, the results assessing concentrations of cytokines IL-17 and IL-23 in laryngeal squamous cell carcinoma should be taken into consideration in the discussion on the role of cytokine activity in the advancement and progression of cancer, its prognosis, and selection of an appropriate therapeutic intervention.

The authors declare no conflict of interest.

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