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ORIGINAL STUDY

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Prognostic value of the immunological phenomena and relationship with clinicopathological characteristics of the tumor — the expression of the early CD69$^+$, CD71$^+$ and the late CD25$^+$, CD26$^+$, HLA/DR$^+$ activation markers on T CD4$^+$ and CD8$^+$ lymphocytes in squamous cell laryngeal carcinoma. Part II

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Abstract: One of the most important challenges in contemporary oncology is to find objective biomarkers of tumor aggressiveness, which help to identify more invasive phenotypes of the carcinoma. The purpose of this study was to investigate the relationships between the early and the late activation markers expression on T CD4$^+$ and CD8$^+$ cells subpopulations and certain clinicopathological characteristics of the neoplastic infiltration in order to determine their role as biomarkers for tumor behavior in squamous cell laryngeal carcinoma. Analysis of the early (CD69$^+$, CD71$^+$) and the late activation antigens (CD25$^+_{high}$, CD26$^+$, HLA/DR$^+$) expression on T CD4$^+$ and CD8$^+$ lymphocytes by cytofluorymetry in 55 patients treated for squamous cell laryngeal carcinoma was performed. Clinicomorphological analysis on the basis of TNM criteria and tumor front grading, which included tumor-related features and adjacent stroma-related characteristics of the peripheral edge of infiltration was carried out. The relationships between the activation markers expression and parameters of tumor aggressiveness were investigated. Our work revealed statistically significant differences in the expression of the studied activation markers on T cells with regard to certain clinicomorphological fetaures. The expressions of CD69$^+$ and CD71$^+$ antigens on T CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ cells as well as CD4$^+$HLA/DR$^+$ markers were higher for pT3 and pT4 tumors, in

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comparison with pT2 carcinomas. Moreover, tumors with the smallest number of TFG points were characterized by significantly lower values of the average expression of $CD3^+CD69^+$ and $CD3^+CD71^+$ as well as $CD4^+HLA/DR^+$ markers on T lymphocytes. In addition, more aggressive and deeply infiltrating laryngeal carcinomas were most often characterized by significantly higher values of the average expression of $CD69^+$ and $CD71^+$ antigens on $CD8^+$ as well as HLA/DR^+ markers on $CD4^+$. Our study confirmed the implication of the early and the late activation antigens expression on $CD4^+$ and $CD8^+$ T lymphocytes in clinicomorphological parameters of the tumor, especially TFG total score and depth of invasion, and their importance as indicators of the invasive phenotype of laryngeal carcinoma. (*Folia Histochemica et Cytobiologica 2011; Vol. 49, No. 4, pp. 593–603*)

Key words: laryngeal carcinoma, T cells activation markers, clinicomorphological features

Introduction

One of the most important challenges in contemporary oncology is to find the objective biomarkers of tumor aggressiveness, which could help to identify more invasive phenotypes of the carcinoma. Defining the biological indicators, explicit in the assessment and interpretation, could allow the selection of tumors characterized by higher progression and thereby to apply an appropriate and optimal treatment as well as to predict the course of neoplastic disease in specific cases of head and neck region cancers.

In the literature concerning this subject, many researchers have pointed to the important role of immune system disturbances in cancer patients [1–7]. In recent years, various research centers have performed multi-experimental and clinical studies on the regulatory mechanisms that determine the activity of immune system cells in response to tumor cells of different origins. Understanding these immunological phenomena could have a significant impact on the suppression of cancer progression in the future. Studies on the effects of immunocompetent cells involved in antitumor response to neoplastic infiltration and the role of activation of T $CD4^+$ and $CD8^+$ lymphocytes in determining tumor invasiveness, as well as the clinical course of neoplastic disease, are currently prominent in clinical immunopathology [3, 8–11].

The purpose of this study was to investigate the relationships between the early ($CD69^+$, $CD71^+$) and the late ($CD25^+_{high}$, $CD26^+$, HLA/DR^+) activation antigens expression on T $CD3^+CD4^+$ and $CD3^+CD8^+$ cells subpopulations and clinicopathological characteristics (pTNM classification, tumor-related and adjacent stroma-related morphological features, TFG total score) of the neoplastic infiltration in order to determine their role as biomarkers for tumor behavior in squamous cell laryngeal carcinoma.

Material and methods

Tissue samples, histological classification and morphological features. For this study, archival paraffin-embedded tissue samples from 55 patients (53 men, two women, aged 48–83 years, mean age 58.3 ± 9) surgically treated for laryngeal squamous cell carcinoma were utilized. Each patient had undergone complete (60%; 33/55) or partial (40%; 22/55) surgical resection of the larynx. 40% (22/55) of the patients underwent dissection of the cervical lymph nodes; selective neck dissection (SND) in 36.4% (20/55) of cases; and radical neck dissection (RND) in 3.6% (2/55) of cases. The lesions were assessed according to the criteria of the International Union Against Cancer (UICC-TNM 2009) for head and neck carcinomas [12]. Morphological estimation was performed on H&E-stained sections in accordance with tumor front grading (TFG) classification [13, 14]. Tumor-related features (infiltration cytoplasmic differentiation, nuclear polymorphism, number of mitoses) and adjacent stroma-related characteristics of the peripheral edge of tumor infiltration (mode of infiltration, depth of invasion and plasmalymphocytic infiltration) in the most invasive, peripheral zones of the neoplasm were analyzed. These factors were assessed in at least five different regions of the peripheral part of the tumor (magnification × 200, number of mitoses magnification × 400). Each factor was graded according to a scale ranging from 1 to 4. The total morphological TFG score was computed as the sum of six parameters, with a maximum score of 24 points. According to the results, tumors were divided into five groups: 6–9, 10–13, 14–17, 18–21 and > 22 TFG points. The histological grade of differentiation, G, was measured according to the generally-accepted three-grade morphological system: G1 (low grade), G2 (intermediate/moderate grade), and G3 (high grade).The observation period of patients after surgical treatment was at least 24 months (from 24 to 54 months). There were no deaths among the patients included in the research. Local recurrence of cancer was found in 5.4% (3/55) of patients 6–18 months after a partial laryngectomy, in whom the treatment of choice was total laryngectomy.

FACS analysis of early and late activation antigens on T $CD4^+$ and $CD8^+$ lymphocytes. The investigations were performed with the approval of the Ethical Committee of the Medical University of Lodz, Poland and the National Science Council, Poland (No RNN/15/03/KN). Blood was collected directly before premedication into pyrogen free Heparin Li-tubes (final concentration 10 U/mL) and resuspend-

ed at a concentration of 1×10^6 cells/mL in RPMI 1640 medium (Biomed, Poland) supplemented with antibiotics streptomycin/penicillin/gentamycin 1% v/v (Sigma, Aldrich, Germany). Next, blood was incubated in 24-well flat-bottomed plates (Nunc Corp., Roskilde, Denmark) in a final volume of 0.2 mL (per well) and collected after 24 h at 37°C, 5% CO_2 (Cellstar Incubator). The experiences with the use of mitogenic stimulation with 5 μg of PHA (phytomagglutinin) were also perfomed. For immunostaining, the following conjugated antibodies were used: anti-CD4 FITC labeled (clone RPA-T4), anti-CD4 PE (SK3), anti-CD8 PE (RPA-T8), anti-CD69 APC (L78), anti-CD71 APC (M-A712), anti-CD25 PE (2A3), anti-CD26 PE (L272) and anti-HLA-DR APC (L243), all provided by BD Pharmingen. 100 ml of blood was mixed and incubated for 30 min. at room temperature with appropriate quantities of antibodies or isotype controls. Erythrocyte contamination was eliminated by the addition of lysing solution (BD Bioscience) into the samples. After brief incubation and rinsing, the samples were fixed with 1% paraformaldehyde and analyzed by flow cytometry (FACSCalibur TM, CELLQuestTM software; BD Bioscience). The cell analysis and gates were restricted to lymphocytes in dot-plot. The results were expressed as mean fluorescence intensity (MFI) of the labeled surface antigens or percent positive $CD4^+$ or $CD8^+$ cells.

Statistical analysis of data. The statistical calculations were made using STATISTICA version 9.0 (StatSoft, Poland). None of the parameters recorded in material studied passed tests for being normally distributed (Kolmogorov–Smirnov test). Kruskal–Wallis one-way ANOVA test as nonparametric analysis of variance by ranks and *post-hoc* tests (Mann–Whitney U test and Dunnett correction for multiple comparisons) for relationships between the activation antigens expression and clinicopathologic parameters were used; p-values ≤ 0.05 were considered to be significant.

Results

Clinicomophological characteristics of group studied. The early activation antigens ($CD69^+$ and $CD71^+$) and the late activation markers ($CD25^+_{high}$, $CD26^+$, HLA/DR^+) activation antigens expression on T cells

To begin with, we studied the morphological features according to pTNM criteria and tumor front grading (TFG) classification in the archival paraffin-embedded tissue samples of squamous cell laryngeal carcinomas. In this study, 25.4% (14/55) of all tumors were classified as pT2 stage, 38.2% (21/55) as pT3, and 36.4% (20/55) as pT4. Nodal stage was histologically assessed as pN0 in 70.9% (39/55) of cases, and as pN1-3 in the remaining 29.1% (16/55) of cases. Pathological analysis of TFG characteristics and the total score for tumors studied was 6–9 points in 9.1% (5/55) of cases, 10–13 points in 30.9% (17/55) of carcinomas, 14–17 points in 41.8% (23/55) of cases and 18–20 points in 18.2% (10/55) of cases, respectively. There were no tumors with TFG score > 21 points in the studied group. Tumors characterized by poor cytoplasmic differentiation (5–20% keratinized) — 36.4% (20/55) of cases, moderate nuclear polymorphism (50–75% mature cells) — 45.5% (25/55), single number of mitoses (0–1) — 56.4% (31/55) as well as neoplastic invasion deeper than submucosa into cartilage (19/55, 34.5%) and tumors with no distinct borderlines (16/55, 29.1%) were the most numerous groups of laryngeal carcinomas studied. The distribution of tumor-related and adjacent stroma-related morphological features, according to TFG classification in the group studied, is shown in Table 1.

The analysis showed that in the studied group mean values of $CD69^+$ and $CD71^+$, the early activation antigens expression on T cells subpopulations, measured as a percentage of T cells with positive expression, were (% ± SEM): 11.1 ± 1.91 for $CD69^+$ and 6.4 ± 1.31 for $CD71^+$ on $CD3^+CD4^+$ T cells and 15.1 ± 2.25 for $CD69^+$

Table 1. Distribution of tumor-related and adjacent stroma-related morphological features, according to TFG classification in the group studied

TFG feature	Characteristic	n (%)
Cytoplasmic differentiation	High (> 50% keratinized)	8 (14.5)
	Moderate (20–50%)	17 (30.9)
	Poor (5–20%)	20 (36.4)
	None (< 5%)	5 (9.1)
Nuclear polymorphism	High (> 75% mature cells)	18 (32.7)
	Moderate (50–75%)	25 (45.5)
	Poor (25–50%)	11 (20.0)
	None (< 25%)	1 (1.8)
Number of mitoses	Single (0–1)	31 (56.4)
	Moderate number (2–3)	13 (23.6)
	Large number (4–5)	7 (12.7)
	Very numerous (> 5)	4 (7.3)
Mode of invasion	Well-defined borderline	12 (21.8)
	Less marked borderline	15 (27.3)
	No distinct borderline	16 (29.1)
	Diffuse growth	12 (21.8)
Depth of invasion	Carcinoma *in situ* (CIS)	6 (10.9)
	Microinvasion into submucosa	12 (21.8)
	Nodular into submucosa	18 (32.7)
	Invasion of cartilage	19 (34.5)
Plasma-lymphocytic invasion	Marked (continuous rim)	7 (12.7)
	Moderate (many large patches)	18 (32.7)
	Slight (few small patches)	24 (43.7)
	None	6 (10.9)

and 4.3 ± 0.89 for $CD71^+$ on $CD3^+CD8^+$ subpopulation, respectively. The evaluation of $CD25^+_{high}$, $CD26^+$, HLA/DR^+, the late activation markers expression on T cells demonstrates the following results: 10.4 ± 1.4 for $CD25^+_{high}$, 0.3 ± 0.05 for $CD26^+$ and 25.4 ± 2.17 for HLA/DR^+ on T $CD3^+CD4^+$ lymphocytes and 9.9 ± 1.14 for $CD25^+_{high}$, 20.8 ± 2.35 for $CD26^+$ and 38.4 ± 2.05 for HLA/DR^+ on T $CD3^+CD8^+$ cells, respectively.

The evaluation of activation markers expression in experiments with mitogenic stimulation demonstrated the presence of the following mean values of the early antigens on $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells (% ± SEM): 32.9 ± 3.6 for $CD69^+$ and 27.9 ± ± 3.64 for $CD71^+$ on $CD3^+CD4^+$ T cells and 45.4 ± 4.08 for $CD69^+$ and 22.8 ± 2.72 for $CD71^+$ on $CD3^+CD8^+$ subpopulation, respectively. The evaluation of the late activation markers expression on T cells demonstrated the following results: 14.9 ± 1.4 for $CD25^+_{high}$, 4.6 ± 0.94 for $CD26^+$ and 34.9 ± 2.31 for HLA/DR^+ on T $CD3^+CD4^+$ lymphocytes and 13.0 ± 1.83 for $CD25^+_{high}$, 26.5 ± 2.54 for $CD26^+$ and 45.2 ± 2.35 for HLA/DR^+ on T $CD3^+CD8^+$ cells, respectively.

Subsequently, we analyzed the relationships of the early ($CD69^+$, $CD71^+$) and the late ($CD25^+_{high}$, $CD26^+$, HLA/DR^+) activation antigens expression with certain clinicopathological features with regard to degree of tumor aggressiveness to evaluate their possible role as potential biomarkers for tumor behavior in squamous cell laryngeal carcinoma.

The relationships between the early ($CD69^+$, $CD71^+$) activation markers expression on T $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes and clinicopathological parameters

To investigate whether the early activation markers such as $CD69^+$ and $CD71^+$ on T lymphocytes can potentially determine clinicopathological features, the cytofluorymetric analysis results were juxtaposed with pathological assessment of the primary tumor (pT status) and the regional lymph nodes (pN status), the histological grade (G), the TFG total score and chosen parameters of TFG classification. The mean expressions of the early activation markers with regard to degree of tumor aggressiveness, according to pT status, TFG classification and the depth of invasion in the trials without/with stimulation, are shown in Table 2.

The evaluation of the early antigens status demonstrated the presence of significant differences in $CD4^+CD69^+$ ($p = 0.04$) and $CD8^+CD69^+$ ($p = 0.01$) expression with regard to pT feature. Data analysis also showed statistically significant differences in the average expression of $CD4^+CD71^+$ ($p = 0.004$) and $CD8^+CD71^+$ ($p = 0.01$) depending on the pT status.

Table 2. Early activation antigens expression on $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells with regard to chosen features

Feature	Characteristic	Without mitogenic stimulation (% ± SEM)			
		$CD4^+CD69^+$	$CD8^+CD69^+$	$CD4^+CD71^+$	$CD8^+CD71^+$
pT	pT2	5.4 ± 1.52	6.4 ± 0.97	2.1 ± 0.43	0.8 ± 0.14
	pT3	13.6 ± 3.37	21.5 ± 4.19	8.4 ± 2.45	6.4 ± 1.74
	pT4	14.3 ± 4.24	15.6 ± 3.32	8.7 ± 2.64	5.4 ± 1.17
TFG score	6–9 points	5.6 ± 2.16	6.2 ± 0.41	2.6 ± 0.61	1.2 ± 0.22
	10–13 points	8.1 ± 2.75	9.0 ± 1.71	4.3 ± 1.82	2.4 ± 0.90
	14–17 points	16.6 ± 3.51	24.6 ± 4.07	10.6 ± 2.37	7.8 ± 1.68
	18–21 points	7.1 ± 1.13	11.6 ± 6.84	2.0 ± 0.75	1.9 ± 0.73
Depth of invasion	Carcinoma *in situ* (CIS)	4.9 ± 2.51	5.5 ± 1.05	2.2 ± 0.81	0.9 ± 0.29
	Microinvasion	10.9 ± 3.13	17.5 ± 5.93	7.6 ± 2.96	5.5 ± 2.31
	Nodular into submucosa	13.6 ± 4.57	14.9 ± 2.96	7.5 ± 2.72	4.4 ± 1.43
	Invasion of cartilage	10.7 ± 2.59	16.8 ± 3.61	5.3 ± 1.27	4.3 ± 1.07
Feature	**Characteristic**	**With mitogenic stimulation (% ± SEM)**			
		$CD4^+CD69^+$	**$CD8^+CD69^+$**	**$CD4^+CD71^+$**	**$CD8^+CD71^+$**
pT	pT2	28.1 ± 5.74	42.8 ± 6.56	22.1 ± 4.69	15.3 ±3.62
	pT3	38.1 ± 5.79	49.8 ± 7.09	29.9 ± 5.87	28.2 ± 4.67
	pT4	30.3 ± 7.62	41.1 ± 7.50	32.3 ± 9.19	23.2 ± 4.88
TFG score	6–9 points	31.3 ± 10.41	47.3 ± 12.76	30.6 ± 10.44	18.1 ± 9.54
	10–13 points	32.6 ± 5.99	40.4 ± 6.01	22.6 ± 5.71	16.6 ± 3.51
	14–17 points	37.2 ± 6.09	50.5 ± 7.38	36.6 ± 5.77	32.1 ± 4.14
	18–21 points	17.4 ± 0.95	41.9 ± 11.16	9.4 ± 1.06	14.9 ± 5.61
Depth of invasion	Carcinoma *in situ* (CIS)	20.1 ± 11.16	37.1 ± 14.29	23.6 ± 10.70	17.7 ± 9.61
	Microinvasion	35.8 ± 5.82	44.6 ± 5.88	23.7 ± 5.63	20.4 ± 5.25
	Nodular into submucosa	37.6 ± 7.76	50.7 ± 8.69	37.9 ± 8.07	26.4 ± 4.89
	Invasion of cartilage	29.1 ± 5.03	43.1 ± 7.34	21.4 ± 4.05	23.1 ± 4.73

The expression of $CD69^+$ and $CD71^+$ antigens on T $CD3^+CD4^+$ and $CD3^+CD8^+$ cells was higher for pT3 and pT4 tumors, in comparison with pT2 carcinomas. No significant differences between the mean expression of early activation markers comparing tumors in various stages of pN and histological differentiation were noted.

We discovered statistically significant differences between mean values of $CD69^+$ ($p = 0.003$) and $CD71^+$ ($p = 0.01$) antigen expression on T $CD8^+$ cells subpopulation taking into account the degree of tumor invasiveness defined by TFG total score. Carcinomas with the smallest number of TFG points (6–9 points) were characterized by significantly lower values of the average expression of $CD8^+CD69^+$ and $CD8^+CD71^+$ antigens. Our results also indicated a significant difference between $CD69^+$ ($p = 0.006$) and $CD71^+$ ($p = 0.01$) antigens on T $CD3^+CD8^+$ cells with regard to depth of invasion. Tumors with noninvasive growth (CIS) demonstrated significantly lower values of average percentage of the early activation markers on T $CD8^+$ lymphocytes subpopulation.

In contrast, more aggressive and deeply infiltrating laryngeal carcinomas were most often characterized by significantly higher values of the average expression of $CD8^+CD69^+$ and $CD8^+CD71^+$ antigens. No significant differences between the mean expression of early activation markers comparing tumors with various types of invasion and the intensity of plasmalymphocytic infiltration were disclosed. Dot plot histograms of representative results of $CD8^+CD69^+$ and $CD8^+CD71^+$ antigens with regard to depth of invasion are shown in Figure 1A–D. The mean expressions of the early activation markers and the statistical test results depending on TFG and the depth of invasion are shown in Figure 2.

No significant relationships between the mean expression of the early antigens and the late activation markers in relation to clinicopathological characteristics in the trials with stimulation were noted.

The relationships between the late ($CD25^+_{high}$, $CD26^+$, HLA/DR^+) activation antigens expression on T $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes and clinicopathological parameters

To check whether the late activation molecules such as $CD25^+_{high}$, $CD26^+$, HLA/DR^+ on T $CD3^+CD4^+$ and $CD3^+CD8^+$ cells could be associated with clinicomor-

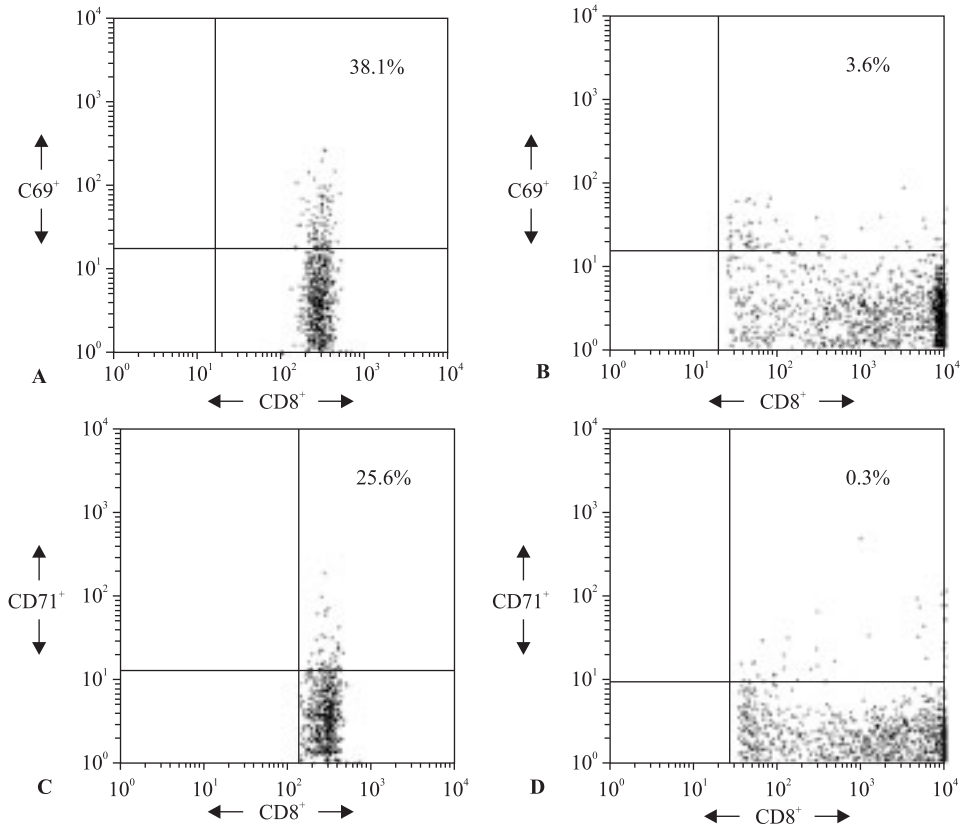

Figure 1. Dot plot histograms of representative results of $CD8^+CD69^+$ and $CD8^+CD71^+$ antigens with regard to depth of invasion (**A**) Higher expression of $CD8^+CD69^+$ (38.1%) for tumors with invasion of cartilage were demonstrated. (**B**) Lower expression of $CD8^+CD69^+$ (3.6%) for CIS was noted. (**C**) Higher expression of $CD8^+CD671^+$ (25.6%) for tumors with invasion of cartilage was disclosed. (**D**) Lower expression of $CD8^+CD71^+$ (0.3%) for CIS was noted

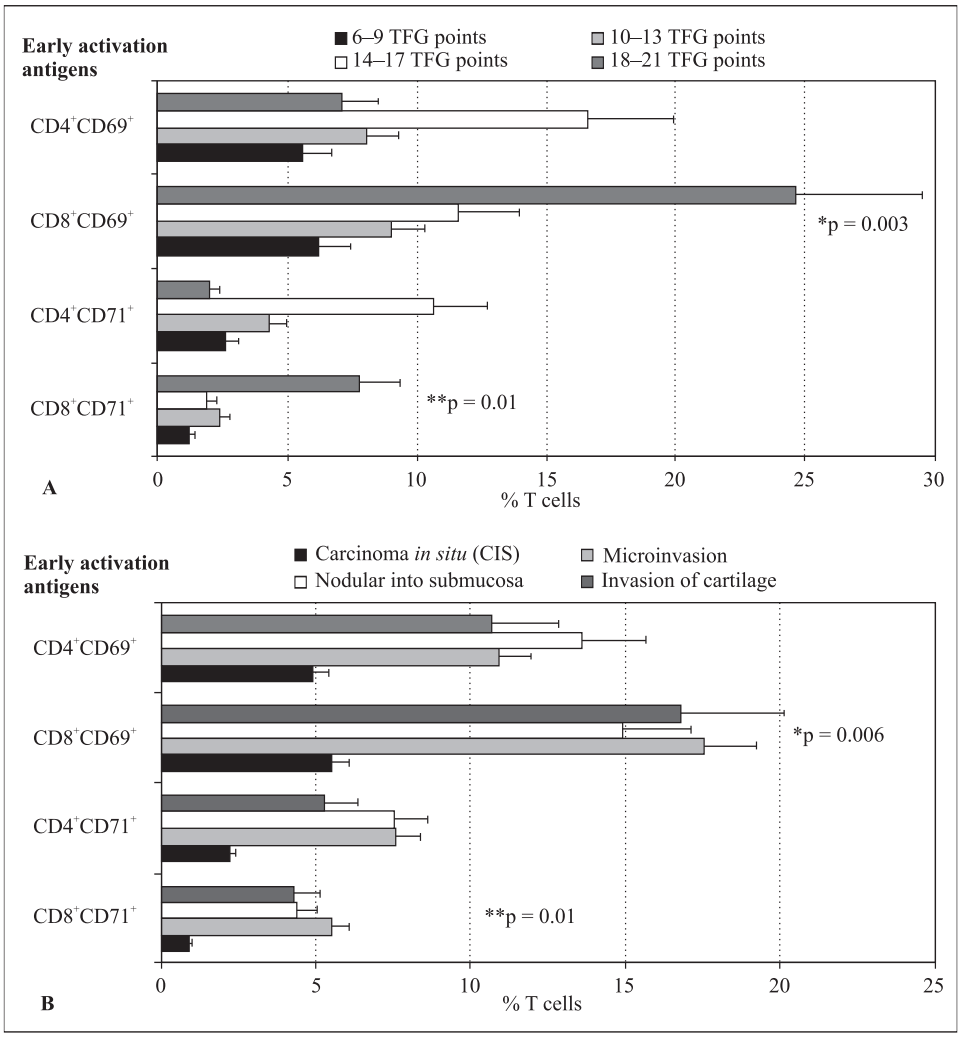

Figure 2. Mean expressions of early activation markers and statistical test results with regard to (**A**) TFG and (**B**) the depth of invasion in tumor group studied

phological parameters, we rated the cytofluorymetric assessment against pT and pN status, the histological grade (G), the TFG total score and chosen parameters of TFG classification. The mean expressions of the late activation markers with regard to degree of tumor aggressiveness, according to pT, TFG classification and the depth of invasion in the trials without/with stimulation, are shown in Table 3.

Data study showed statistically significant differences in the average expression of HLA/DR$^+$ antigens on T CD4$^+$ cells depending on the pathological assessment of the primary tumor (pT status) ($p = 0.009$). Carcinomas with the highest local stage pT4 were characterized by significantly higher values of the average level of CD4$^+$HLA/DR$^+$. No significant differences between the mean expression of the other late activation markers comparing tumors in various stages of pN and histological differentiation were noted.

The evaluation of the late antigens status also demonstrates the presence of significant differences in CD4$^+$HLA/DR$^+$ ($p = 0.04$) expression with regard to various degree of tumor aggressiveness, according to TFG total score. Carcinomas with the smallest number of TFG points (6–9 points) were characterized by significantly lower values of the average expression of HLA/DR$^+$ antigens on T CD4$^+$ lymphocytes. In addition, we confirmed significant differences of the late activation markers between various depths of tumor invasion. Patients with carcinomas characterized by the lowest values of the average expression of CD4$^+$HLA/DR$^+$ on T cells were found to demonstrate the least invasive grow. No significant differences between the mean expression of early activation markers comparing tumors with various types of invasion and the intensity of plasmalymphocytic infiltration were noted. Dot plot histograms of representative results of CD4$^+$HLA/DR$^+$ antigens with

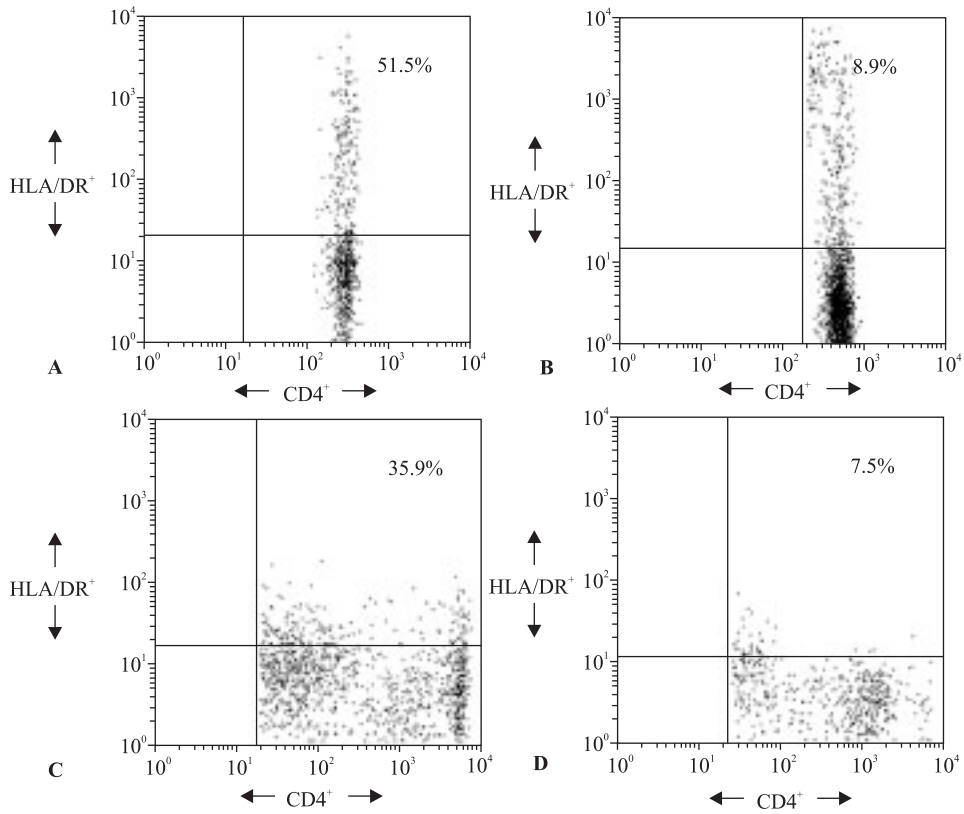

Figure 3. Dot plot histograms of representative results of $CD4^+HLA/DR^+$ antigens depending on pT status and depth of invasion (**A**) Higher expression of $CD4^+$ HLA/DR^+ (51.5%) for pT4 tumors was demonstrated. (**B**) Lower expression of $CD4^+$ HLA/DR^+ (8.9%) for pT2 was noted. (**C**) Higher expression of $CD4^+HLA/DR^+$ (35.9%) for tumors with invasion of cartilage was disclosed. (**D**) Lower expression of $CD4^+HLA/DR^+$ (7.5%) for CIS was noted

regard to TFG score and the depth of invasion are shown in Figures 3A–D. The mean expressions of the late activation markers and the statistical test results depending on TFG score and the depth of invasion are shown in Figure 4.

Our data demonstrated a statistically significant increase in the average expression of $CD4^+HLA/DR^+$ activation markers when laryngeal cancers were more extensive and pT status was higher, in the experiments with mitogenic stimulation ($p = 0.04$). Increased expression of HLA/DR^+ antigens on T $CD3^+CD4^+$ cells was the highest in pT3 and pT4 tumors. We did not discover statistically significant differences between mean values of other antigens expression on T cells subpopulations in relation to clinicopathological parameters in the trials with stimulation.

Discussion

This study found that laryngeal squamous cell carcinomas in a high local stage of neoplastic lesions (pT3 and pT4 tumors), were characterized by a significantly higher expression of the examined early activation antigens, i.e. $CD4^+CD69^+$, $CD8^+CD69^+$, $CD8^+CD71^+$, $CD8^+CD71^+$ in comparison with pT2 carcinomas.

Among the analyzed markers of late activation, only HLA/DR^+ antigens on T $CD4^+$ lymphocytes showed higher expression in advanced cancers of the larynx. It was also shown that tumors with the least invasive changes, such as cancer with the smallest number of points referred to the criteria of TFG classification, were characterized by the lowest expression of the early antigens $CD69^+$ and $CD71^+$ on T $CD8^+$ cells and also the lowest expression of the late activation markers HLA/DR^+ on T $CD4^+$ cells, in experiments without mitogen stimulation. The percentage of T $CD4^+$ and $CD8^+$ cells in circulating blood and the ratio of $CD4^+/CD8^+$ was not significantly associated with analyzed clinicopathological parameters. The results obtained in our study indicate the occurrence of immune activation of T cells in peripheral blood, both T $CD4^+$ and $CD8^+$ cells in the early phase of immune response to the presence of tumor antigens in cases of laryngeal cancers in high-stage changes. However, the observed absence of relationships of the late activation markers expression on T cells, in particular on $CD8^+$ subpopulations with clinicomorphological features in invasive carcinomas of the larynx, indicates a progressive disturbance and decrease in the effectiveness of antitumor immune

Table 3. Late activation antigens expression on $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells with regard to chosen features

Feature	Characteristic	Without mitogenic stimulation (% ± SEM)					
		$CD4^+CD25^+_{high}$	$CD8^+CD25^+$	$CD4^+CD26^+$	$CD8^+CD26^+$	$CD4^+HLA/DR^+$	$CD8^+HLA/DR^+$
pT	pT2	9.7 ± 1.84	10.8 ± 2.01	0.2 ± 0.02	22.7 ± 2.27	16.8 ± 2.68	32.1 ± 2.74
	pT3	9.1 ± 1.98	8.5 ± 1.88	0.3 ± 0.10	20.2 ± 4.03	27.7 ± 3.28	42.9 ± 3.08
	pT4	13.5 ± 3.93	10.7 ± 2.31	0.4 ± 0.08	20.6 ± 4.32	32.9 ± 3.88	39.3 ± 4.48
TFG score	6–9 points	11.7 ± 4.56	6.7 ± 4.30	0.2 ± 0.05	25.4 ± 3.45	18.2 ± 1.91	32.5 ± 6.28
	10–13 points	11.2 ± 1.98	12.0 ± 1.17	0.2 ± 0.03	23.2 ± 4.96	20.7 ± 3.38	36.2 ± 12.25
	14–17 points	7.4 ± 0.94	10.3 ± 2.30	0.4 ± 0.09	17.7 ± 3.09	32.8 ± 2.98	43.1 ± 10.71
	18–21 points	17.8 ± 11.36	4.6 ± 1.05	0.5 ± 0.01	31.5 ± 5.01	22.2 ± 9.12	35.4 ± 11.76
Depth of invasion	Carcinoma *in situ* (CIS)	9.5 ± 2.60	9.0 ± 3.38	0.2 ± 0.01	28.9 ± 0.01	16.0 ± 3.09	32.4 ± 3.21
	Microinvasion	11.2 ± 2.43	9.6 ± 2.38	0.3 ± 0.09	24.3 ± 5.88	22.1 ± 4.08	37.7 ± 4.22
	Nodular into submucosa	8.5 ± 2.02	9.0 ± 2.70	0.3 ± 0.14	14.6 ± 2.52	28.1 ± 4.33	42.1 ± 3.82
	Invasion of cartilage	12.3 ± 4.06	11.3 ± 1.87	0.4 ± 0.07	23.2 ± 3.44	30.2 ± 3.11	37.1 ± 3.71
Feature	**Characteristic**	**With mitogenic stimulation (% ± SEM)**					
		$CD4^+CD25^+_{high}$	$CD8^+CD25^+$	$CD4^+CD26^+$	$CD8^+CD26^+$	$CD4^+HLA/DR^+$	$CD8^+HLA/DR^+$
pT	pT2	16.2 ± 1.80	12.2 ± 1.90	5.0 ± 1.90	31.9 ± 2.95	27.8 ± 3.51	40.5 ± 4.08
	pT3	12.7 ± 2.03	10.9 ± 3.43	5.1 ± 1.73	23.9 ± 4.01	36.2 ± 3.59	47.7 ± 3.78
	pT4	17.0 ± 3.35	17.4 ± 4.38	3.6 ± 0.63	27.1 ± 4.87	42.5 ± 4.04	47.0 ± 4.29
TFG score	6–9 points	17.9 ± 3.50	7.5 ± 4.25	6.8 ± 3.85	36.1 ± 2.80	27.6 ± 3.20	42.4 ± 13.41
	10–13 points	15.9 ± 1.99	13.3 ± 0.88	4.6 ± 1.60	26.5 ± 5.15	34.4 ± 4.64	43.3 ± 13.78
	14–17 points	11.7 ± 1.38	14.2 ± 4.19	4.4 ± 1.39	23.2 ± 3.12	38.6 ± 2.69	49.8 ± 14.06
	18–21 points	20.2 ± 9.37	14.4 ± 12.40	2.6 ± 0.01	42.8 ± 6.01	30.7 ± 9.93	36.7 ± 6.30
Depth of invasion	Carcinoma *in situ* (CIS)	2.6 ± 0.84	9.5 ± 3.17	10.7 ± 0.01	38.9 ± 0.01	22.5 ± 4.76	40.7 ± 7.48
	Microinvasion	3.2 ± 0.65	11.3 ± 2.38	2.1 ± 0.45	28.8 ± 5.32	33.1 ± 3.67	45.2 ± 4.35
	Nodular into submucosa	2.4 ± 0.34	13.6 ± 6.66	3.8 ± 1.48	23.0 ± 3.11	37.7 ± 5.15	48.2 ± 5.01
	Invasion of cartilage	2.8 ± 1.01	16.4 ± 3.53	7.1 ± 2.01	26.2 ± 5.54	39.5 ± 2.50	43.1 ± 2.93

mechanisms, which progressed with increasing time of interaction of tumor cells to immune cells.

The phenomenon of suppressive effects of tumor on the function of cells involved in cellular immune response can lead to anergy and immunological tolerance to foreign antigens in cancer patients. Also important is the role of regulatory lymphocytes, subpopulation of $CD4^+$ cells which, by the immunosuppressive activity directed against the effector cells, also contribute to the inhibition of antitumor immune mechanisms and increasing cancer progression [2, 6, 8, 15–23]. The causes of the dysfunction of T lymphocytes, both $CD4^+$ and $CD8^+$ cells and absence of stimulation of cells participative in the cellular immune response to tumor antigens, also include changes in the level of secreted cytokines, marked in the peripheral blood in patients with laryngeal cancer. A lack of activation of immune mechanisms dependent on IL-6 and IFN-γ results in the reduced effectiveness of cellular immune response to carcinoma antigens. The reduction in IL-6 in the peripheral blood can result in a decrease of the activity of T cells that recognize antigen, impaired differentiation of antigen-stimulated T cells into the cytotoxic lymphocytes, as well as reduction in Tc and NK cells activation and thus direct inhibition of tumor cell proliferation and cytolytic activities [2, 3, 18, 24–27].

No effect of IFN-γ promotes expression of MHC molecules on the APC cells and enhances antigen presentation, increases the cytotoxicity of Tc lymphocytes, induces expression of cytokines such as IL-1α, IL-6, TNF, and thus stimulates the activity of $CD4^+$ and $CD8^+$. This confirms the immunosuppressive effects of tumor cells on T lymphocytes and indicates a defect of the immune response caused by the malfunctioning of immune cells [2, 3, 18, 24–27]. Suppression of immune response in advanced laryngeal cancer increases high level of secreted IL-10, which also inhibits the expression of MHC class II molecules on macrophages and reduces the presentation of tumor antigens as well as inhibits the formation of Th1 cells and cytokine production by Th1 (IFN-γ). The presented observations are therefore evidence of the ineffectiveness of immune mechanisms, dependent on active T lymphocytes and also indicate the efficacy of escape mechanisms used by tumor cells in defense against anti-tumor immune response [2, 3, 18, 24–27].

In the literature, it is difficult to find publications in which the relationship of antigen expression on activated T lymphocytes in laryngeal squamous cell

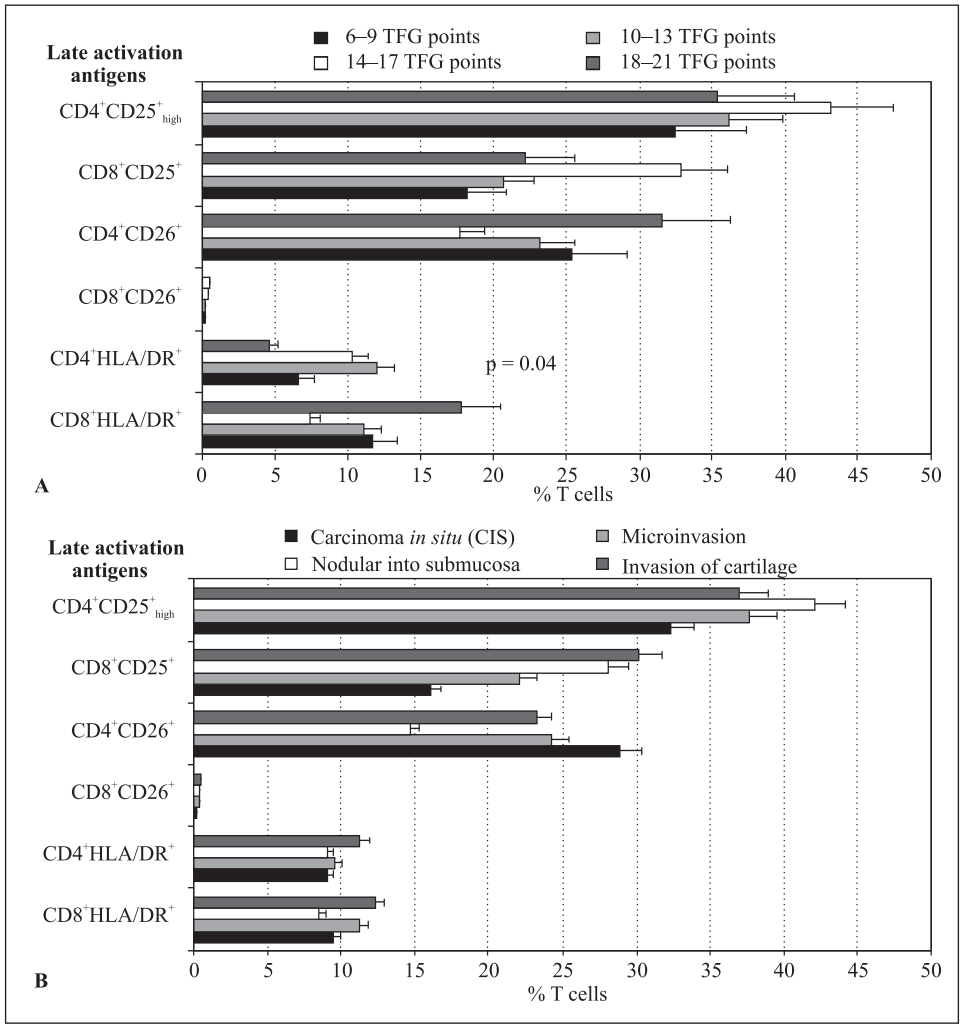

Figure 4. Mean expressions of late activation antigens and statistical test results depending on (**A**) TFG score and (**B**) the depth of invasion in carcinoma group studied

carcinoma in the context of assessing the impact of the activity of immunocompetent cells on the clinicomorphological parameters of the tumor has been analyzed. Moreover, researches on carcinomas of the head and neck region have mostly related to the assessment of expression of the markers on tumor infiltrating cells (TIL), rather than circulating blood cells, which does not allow direct comparison of results [6, 15, 28–30]. For discussion, only publications concerning cancers of the head and neck region, in which the authors had adopted similar techniques to the used panel of activation markers on T cells, and similar research methods, were chosen. In addition, individual research papers in which the relationships between immunological parameters and clinicomorphological characteristics of tumor were analyzed, related only to typical histological markers. In these publications, the criteria for the TNM classification, histological grade G and the degree of clinical progression of the laryngeal carcinoma S often were concerned with prognostic indicators such as survival and tumor recurrence [6, 15, 28–30]. It should be emphasized that the results of many authors show large discrepancies in assessing the activity of cells involved in immunological processes in various types of head and neck carcinomas and lead to different conclusions regarding the relation of activation markers expression on immune cells and clinicomorphological indicators [6, 15, 28–30].

Regardless of these differences in methodology and the importance of heterogeneity in the analyzed groups of cancers, studies on the activity of immune cells, which participate in immunological reactions in the course of tumor progression, indicate varying degrees of intensified suppression of immune cellular mechanisms, evaluated as the activation antigens expression on T $CD4^+$ and $CD8^+$ cells in squamous cell carcinomas of the head and neck [6, 15, 19, 28–31]. The presence of increased suppression of T $CD8^+$ cells in peripheral blood in the most advanced clinical stage (IV S), indicating the abnormal relationship

of effector cells activity in invasive tumors of the head and neck region, was confirmed [29]. Analysis of clinicomorphological markers in another group of patients with HNSCC has indicated a decreased number of T $CD4^+$ and $CD8^+$ cells in circulating blood, without demonstrating the relationships of immune cells activity with the parameters of TNM classification [30]. In other studies, the assessment of activity of T cells subpopulations among tumor infiltrating lymphocytes (TIL), demonstrated the absence of relationships between the expression of $CD4^+$ phenotype with the local extent of tumor pT, nodal stage pN and the primary location of tumor invasion [32]. The dependence of the high percentage of lymphocytes with $CD8^+$ phenotype in peripheral blood and also the presence of a reduced ratio of $CD4^+/CD8^+$, which are associated with increased invasiveness of tumor determined on the basis of pT in patients with tumors of the head and neck, has also been demonstrated [6].

Other authors, however, did not confirm the correlation of $CD4^+/CD8^+$ ratio with progression of neoplastic lesions, treatment outcomes, incidence of tumor recurrences and survival in patients with carcinomas of the larynx [31]. In other studies on the expression of both early antigens $CD69^+$ and $CD71^+$ and the late activation markers $CD25^+$ and $HLA\text{-}DR^+$ on T lymphocytes in peripheral blood in relation to the pTNM classification, there were significant correlations between the analyzed indicators. The authors found that high expression of $CD71^+$ molecules on T $CD3^+$ lymphocytes and a high rate of $CD69^+/CD71^+$ as well as $CD25^+/HLA\text{-}DR^+$ were significantly associated with increasing invasiveness of cancerous changes according to pTNM in tumors of the head and neck [28]. Researchers also point to the importance of the expression of various activation markers in the progression of the neoplastic disease and thus the role of activation antigens as prognostic indicators. There have been no deaths among the patients included in this study. Local recurrence of cancer was found only in three patients 6–18 months after a partial laryngectomy. Analysis of activation antigens expression on T lymphocytes as prognostic indicators has not been studied in this paper, because of the previously described clinical characteristics of the group studied. Nevertheless, the findings of other authors indirectly indicate the role of the degree of immune cells activity in cancer patients as well as the importance in determining the advancement of neoplastic changes. A significant correlation of high percentage of TIL characterized by $CD4^+CD69^+$ phenotype with a lower risk of local recurrence and prolonged survival of patients with head and neck carcinomas has been demonstrated. Interestingly, the increase in the percentage of lymphocytes with $CD4^+Foxp3^+$ phenotype in tumor stroma was also associated with a decreased incidence of tumor recurrence [32]. The pathomechanism leading to the observed changes are explained by the influence of regulatory T cells on T $CD4^+CD25^+Foxp^{3-}$ and $CD8^+$ lymphocyte subpopulations. Treg lymphocytes, which dampen down the activity of T $CD4^+$ cells and $CD8^+$ cytotoxic lymphocytes, leading to inhibition of the inflammatory response, promote tumor growth, and thereby contribute to reducing tumor invasiveness and local recurrences. The researchers also noted a positive correlation between the percentage of T $CD4^+CD69^+$ cells with the number of T cells characterized by $CD4^+CD25^+$ phenotype in tumor stroma, but they did not show such a relationship for T $CD4^+CD69^+$ cells expressing $CD4^+Foxp^{3+}$ antigens CD4. A decreased percentage of TIL $CD4^+$ lymphocytes was associated with an absence of neoplastic recurrences but did not impact on survival. Different results were presented by other investigators who demonstrated a significant correlation of reduced $CD4^+$ antigen expression on peripheral blood lymphocytes, with an increased incidence of local recurrences in cases of head and neck cancers [29, 30]. Other authors have confirmed a positive relationship between the number of tumor infiltrating T lymphocytes which showed $CD25^+$ antigen expression with prolonged survival in the study group [19].

A significant association of early activation antigen $CD71^+$ expression on immune cells in the circulating blood with survival in the studied group of patients with cancers of the head and neck region was also demonstrated. The results indicate the role of $CD71^+$ high expression as a negative prognostic factor [28]. Researchers have also shown a significant correlation between expression of the early and the late activation antigens and survival of patients with HNSCC. High level of the coefficients $CD69^+/CD71^+$ and $CD25^+/HLA\text{-}DR^+$ was associated with poor prognosis in the study group. The discrepancies in the results of studies assessing the role of activation antigens expression on T lymphocytes as indicators of advancement of neoplastic changes, and the prognostic significance of activity of cells involved in immunological processes, indicate a need for further research to obtain clear conclusions concerning the pathomechanism of tumor progression in many cancers of the head and neck, including cancers of the larynx.

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