Expression of CTLA-4 and Foxp3 in peripheral blood T cells of patients with squamous cell laryngeal carcinoma

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Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, CD152) and Foxp3 (forkhead box P3) are receptors present on T cells which play a critical role in the down-regulation of antigen-activated immune responses. To evaluate the potential influences of CTLA-4 and Foxp3 on cancer invasiveness, a case-control study was conducted in 86 patients treated for squamous cell laryngeal carcinoma. The abundance of CTLA-4 and Foxp3 gene transcripts in the purified peripheral blood mononuclear cells (PBMCs) by quantitative real-time PCR (qRT-PCR) was determined. The analysis of proteins by Western blot was performed. The relationships between CTLA-4 and Foxp3 gene and protein expression as well as the aggressiveness of tumor determined on pT, type and depth of invasion were investigated. Our work revealed a significant dependence of mRNA CTLA-4 on tumor front grading (TFG) total score (p = 0.04) as well as CTLA-4 protein expression on pT (p = = 0.03) and type of invasion (p = 0.03). Advanced pT3-pT4 tumors with diffuse infiltration and > 14 TFG points were characterized by higher average values of CTLA-4 protein in PBMCs. Our data also demonstrated significant differences between Foxp3 protein levels in relation to pT (p = 0.04), depth of invasion (p = = 0.02) and type of invasion (p = 0.03). In tumors with the highest invasiveness identified by the pT3-pT4 status, deep invasion with involvement of cartilage and diffuse infiltration, the highest Foxp3 protein level was observed. In conclusion, these results suggest an impact of CTLA-4 and Foxp3 in determining proliferative and aggressive potential of laryngeal carcinoma, highlighting the significance of CTLA-4 and Foxp3 as potential predictive indicators.

Key words: laryngeal carcinoma, CTLA-4 (CD152), Foxp3, peripheral blood T cells, mode and depth of invasion.

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Regulatory T cells (Tregs) play a pivotal role in progression and modulation of the immune escape mechanisms used by cancer cells [1–10]. A lot of data suggest that in patients with neoplastic disease including head and neck carcinomas, there is an increase of $CD4^+CD25^{high}Foxp3^+$ regulatory T-cells either in the peripheral blood or tumor microenvironment [2–5, 7, 9–34]. Therefore, Tregs might play a crucial function in the regulation of the immune response against tumor cells and thus they could be important in the design of immunotherapy [1–35]. The suppressive function of Tregs depends on interactions between stimulatory (IL-2) and inhibitory (GITR, CD28) signals, on stimulation of indoleamine 2,3-dioxygenase (IDO) activity in dendritic cells (DCs) via CD80/CD86 molecules, and finally on cell-cell inhibition of effector cells by secretion of IL-10 and TGF-β1 [1, 2, 12, 14]. In addition, several studies have documented modulation of Th17/Tregs balance and the anergy or suppression of effector cells in cancer disease as a consequence of the Th17-driven procarcinogenic immune response by IL-17A [1, 15]. However, the precise mechanisms of Treg mediated suppression have not yet been fully characterized, but the suggested mediators include CTLA-4 (cytotoxic T-lymphocyte antigen-4, CD152) and Foxp3 (forkhead/winged helix transcription factor). These negative regulatory molecules have been used recently as biomarkers and prognostic factors for malignant tumors of various origin [1–13, 15, 16, 18–20, 22–30, 32–34]. They are important mediators of peripheral immune tolerance, acting via various mechanisms to suppress cellular immunity and thus they are also potential targets for immunotherapy [5, 17, 35]. Nevertheless, little is known on the direct interconnection of the clinicopathological features and prognosis with CTLA-4 and Foxp3 expression or their implications for the corresponding aggressiveness of the tumor in laryngeal carcinoma.

The aim of this study was to assess the CTLA-4 and Foxp3 expression levels in peripheral blood T cells and suppressant role in antitumor response and to evaluate the relationships with clinicomorphological parameters in squamous cell laryngeal carcinoma.

Material and methods

Study subjects

In this study 86 (84 men, 2 women; age 45–79 years; mean age 61.9 ±8 years) patients treated for squamous cell laryngeal carcinoma were analyzed. Each patient underwent complete (73.3%, 63/86) or partial (26.7%; 23/86) surgical resection of the larynx and 53.5% (46/86) of the patients underwent dis-

section of the cervical lymph nodes with pathologic confirmation of metastases (pN1-3) in 25.6% (22/86) of cases. Nodal stage was histologically confirmed as pN0 in 64 (74.4%) cases. The lesions were assessed according to the criteria applied in accordance with the UICC TNM classification of 2003 for head and neck carcinomas [36]. Criteria for patient participation in this study were as follows: 1) a pathologically confirmed diagnosis of carcinoma planoepitheliale, 2) primary surgical resection without receiving prior immuno-, radio- or chemotherapy, 3) absence of distant metastases. In this study 29 (33.7%) of all tumors were classified as stage pT1–pT2 and 57 (69.3%) as pT3–pT4.

Histological classification and morphological features

Morphological evaluation was performed on H&E-stained sections in the peripheral parts of a tumor, according to the histological grade of differentiation and tumor front grading (TFG) classification [37]. The histological grade (G) was measured according to the generally accepted three-grade morphological system: G1 (low grade or well-differentiated tumor), G2 (intermediate/moderate grade or moderately differentiated tumor), G3 (high grade or poorly differentiated tumor). The mode of infiltration and depth of invasion in the most invasive, peripheral zones of the tumor were analyzed. These factors were assessed in at least five different regions of the peripheral part of the tumor (magn ification 200×, number of mitoses magn ification 400×). Type and depth of invasion were graded according to a TFG scale. In addition, tumors were divided into groups (ranks): 6–13 and 14–21 points of TFG. In this study 28 (32.5%) of all tumors were classified as less advanced tumors (6–13 points) and 58 (67.5%) as more aggressive tumors (14–21 points). Type of invasion was histologically assessed as well-defined/less marked borderline cases in 33 (38.5%) cases and as diffuse invasion with no distinct borderline/diffuse growth in 53 (61.5%) tumors. Depth of invasion was pathologically classified as invasion to lamina propria in 21 (24.3%) cases and as invasive infiltration with involvement of muscle and cartilage tissue in 65 (75.7%) cases. Analysis of the primary tumor revealed that a majority of the patients presented carcinoma of intermediate histological differentiation G2 – 67 (77.9%) cases.

PBMC isolation

For isolation of PBMCs (T lymphocytes) the venous blood of each patient was obtained (10 ml) before surgical treatment and transferred to test tubes containing heparin (10 U/ml). PBMCs were isolated by Ficoll-Paque™ PLUS (1.077 density) and resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 medium. The recovered cells were checked and counted for viability with the trypan blue staining method. The isolated cells were collected immediately after the procedure and frozen at –70°C. The control blood samples were obtained from 70 healthy volunteers without a history of malignancies or autoimmune disorders. 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/13/11/KE).

Total RNA extraction and cDNA synthesis

The total RNA was extracted using TRI Reagent (Sigma Aldrich, USA) according to the manufacturer's protocol. RNA was diluted in 20 µl RNase-free water, quantified by spectrophotometry at 260 nm and stored at –20°C. RNA with a 260/280 nm ratio in the range 1.8–2.0 was considered high quality. First-strand cDNA was synthesized from each RNA pool using PCR Kit ver. 3.0 (Takara Bio Inc., Japan) according to the manufacturer's instructions. Briefly, 1 µg of RNA was combined with 2.5 pmol of oligo dT-adapter primer, 4 µl of 25 mM $MgCl_2$, 2 µl 10 × RNA PCR buffer, 2 µl of 10 mM dNTP mixture, 20 units of RNase inhibitor, 5 units of AMV Reverse Transcriptase XL, and RNase-free water to a total volume of 20 µl. The reaction took place at 42°C for 30 min, followed by 95°C for 5 min and 5°C for 5 min in a GeneAmp PCR System 9700 (Perkin-Elmer Co, USA). cDNA was stored at –20°C.

Real-time quantitative PCR (qRT-PCR)

The real-time PCR was performed in a Mastercycler ep Realplex 4S (Eppendorf, Germany). Quantitative evaluation of CTLA-4, Foxp3 and HPRT1 (hypoxanthine phosphoribosyltransferase 1), as a control reference gene was performed with commercially available TaqMan probes CTLA-4 – Hs03044418_m1 (95 bp), Foxp3 – Hs01085835_m1 (107 bp) and Hs02800695_m1, respectively (Applied Biosystems, USA). PCR reactions were carried out in a total volume of 10 µl, containing 0.5 µl of respective TaqMan probes, 3.5 µl of nuclease free water, 5 µl of universal master mix (Applied Biosystems, USA) and 1 µl of cDNA. The reactions were performed in duplicate. Abundance of studied genes' mRNA in samples was quantified by the ΔC_t method. Ct (Ct_{gene}–Ct_{HPRT1}) values were recalculated into relative copy number values (number of copies of studied gene mRNA per 1000 copies of HPRT1 mRNA).

Immunoblotting analysis

Cytoplasmic and nuclear fractions were prepared separately from each sample by differential centrifugation of tissue homogenate in 0.25 M sucrose in buffer containing 5 mM MgCl2, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 50 mM Tris-HCl at pH 7.4, in the presence of 10 mM sodium molybdate. After centrifugation at 800 xg for 7 min the crude nuclear pellet was purified by centrifugation through 2.2 M sucrose in the above buffer at 40 000 xg for 60 min. Supernatant from the first spin, corresponding to the cytoplasmic fraction, was centrifuged at 1500 xg for 10 min to remove any remaining nuclei. Protein concentration in cellular fractions was estimated by means of the modified Lowry procedure [38] using bovine serum albumin as a standard. Cytoplasmic and nuclear fraction proteins (50 µg) were resolved on 8% SDS-polyacrylamide slab gel [39] and electrotransferred onto Immobilon P membrane (Millipore Corp. Bedford, USA) using semi-dry technique [40]. After blocking in 0.5% bovine serum albumin the membrane was incubated with anti-CTLA-4 (sc-9094, H-126) and anti-Foxp3 (sc-28705, H-190) antibody (Santa Cruz Biotechnology, USA). Following extensive washing with TBST buffer (Tris-buffered saline with Tween 20) the membrane was incubated with biotinylated mouse anti-rabbit IgG-HRP (sc-2357) antibody

(Sigma Chemical Co., USA). Specificity of antigen-antibody interaction was tested by streptavidin/biotinylated horseradish peroxidase complex (Strept ABComplex/HRP) (Dako A/S, Denmark) and visualized with 4-chloro-1-naphthol and hydrogen peroxide as a substrate for HRP.

Qualitative and quantitative estimations of immunoblots

For qualitative and quantitative analysis of immunoblots, a video densitometer (Biotec-Fischer, Germany) and software Gel-Pro® Analyzer 3.0 (Media Cybernetics, USA) were used. The integrated optical density (IOD) of the bands, in a digitalized picture, was measured. For the immunoblot analysis the densitometric data of each sample were analyzed based on IOD and were expressed using a three-point scale corresponding to the densitometric ranges: no staining (IOD = 0), moderate staining (IOD > 0–0.4), strong staining (IOD > 0.4).

Statistical analysis of data

Statistical analysis was performed using STATISTICA version 9.0 (StatSoft, Poland). ΔCt values (which have a symmetrical theoretical distribution) obtained from quantitative real-time PCR were recalculated into relative copy number values. Obtained results were not normally distributed (Kolmogorov-Smirnov test) and therefore nonparametric statistical tests were used for analyzing the results (Mann-Whitney U test and Kruskal-Wallis test). A value of $p < 0.05$ was considered statistically significant.

Results

Relationships of CTLA-4 expression with pT status, type and depth of invasion and TFG total score

Our study confirmed mRNA CTLA-4 positive expression in 94.2% (81/86) of all PBMC samples from individuals with SCLC and in 97.1% (68/70) of control PBMC samples. In these groups studied the mean values of mRNA CTLA-4 were 70.42 ±85.56 copies of CTLA-4 mRNA per 1000 copies of HPRT1 mRNA and 16.23 ±16.81 copies of CTLA-4 mRNA per 1000 copies of HPRT1 mRNA, respectively. Significant differences in mRNA CTLA-4 expression between SCLC PBMCs and non-cancerous PBMCs ($p < 0.0001$) were observed. Positive CTLA-4 protein nuclear fraction in 77.9% (67/86) of all PBMC samples from individuals with SCLC and in 77.1% (54/70) of control PBMC samples were assessed. The mean values of CTLA-4 protein nuclear level in both types of samples were 41.47 $\pm 37.53_{IOD}$ and 34.80 $\pm 34.72_{IOD}$, respectively. Significant differences between CTLA-4 protein nuclear expression in SCLC PBMCs and control PBMCs ($p = 0.75$) were not confirmed. Positive protein cytoplasmic fraction of CTLA-4 in 4.6% (4/86) of cancerous PBMCs was observed. In no case of the control group was a CTLA-4 protein cytoplasmic fraction assessed. The mean value of CTLA-4 cytoplasmic level in cancerous samples was 32.45 $\pm 23.34_{IOD}$. Subsequently, the expression pattern of the gene and protein of CTLA-4 in isolated PBMCs from SCLC patients with clinicomorphological parameters was compiled. Statistical evaluation of the quantitative analysis results and the clinicomorphological features of laryngeal carcinomas showed that the expression of mRNA for CTLA-4 in PBMCs isolated from SCLC patients was significantly different depending on the total score of tumor front grading classification ($p = 0.04$). The presence of a higher content of mRNA CTLA-4 was more frequent for tumors with more aggressive behavior determined by higher total score of the TFG scale and characterized by 14–21 points (200.49 ±146.12 copies of CTLA-4 mRNA per 1000 copies of HPRT1 mRNA) in comparison with less invasive carcinomas not exceeding 6–13 points in TFG (143.89 ±148.11 copies of CTLA-4 mRNA per 1000 copies of HPRT1 mRNA). The statistical analysis did not confirm the presence of significant differences in the level of mRNA expression of CTLA-4 in PBMCs with regard to the pT status, tumor growth and type of invasion. However, there can be noted a clear tendency to higher values for mRNA CTLA-4 in laryngeal carcinomas characterized by the indistinct tumor front borderlines and more aggressive behavior determined by muscle tissue and cartilage invasion. Moreover, in pT3–pT4 tumors the mean values of CTLA-4 protein nuclear fraction were lower (46.22 $\pm 37.96_{IOD}$) in comparison with less advanced carcinomas (pT1–pT2) characterized by higher mean values of CTLA-4 (78.05 $\pm 64.46_{IOD}$). The statistical analysis confirmed the presence of significant differences in the level of CTLA-4 in SCLC PBMCs between pT1-pT2 and pT3-pT4 tumors ($p = 0.03$). Well-defined and less marked borderlines of tumor infiltration were characteristic for carcinomas with lower mean values of CTLA-4 in positive PBMCs (46.55 $\pm 38.98_{IOD}$). In contrast, diffuse growth and indistinct tumor front borderlines were most often seen in SCLC characterized by higher mean values of CTLA-4 (77.15 $\pm 63.98_{IOD}$) in PBMCs. Unfortunately, for other clinicopathological parameters statistically significant differences were not disclosed. However, there can be noticed a tendency to higher values for CTLA-4 in laryngeal carcinomas with the aggressive tumor growth determined by the cartilage invasion and high tumor front grading. CTLA-4 expression with regard to the clinicopathological features (pT stage, depth of invasion, mode of invasion and the tumor front grading total score) in SCLC PBMCs is shown in Fig. 1A–H. Because of the small percentage of PBMCs with a positive protein cytoplasmic fraction of CTLA-4, statistical analysis was not performed in the group studied. It should be noted, however, that all cases of SCLC with positive CTLA-4 cytoplasmic expression were characterized by more aggressive behavior.

Relationships of Foxp3 expression with pT status, type and depth of invasion and TFG total score

mRNA Foxp3 positive expression in 72.1% (62/86) of all PBMC samples from patients with SCLC and in 71.4% (50/70) of control PBMC samples was noted. In groups studied the mean values of mRNA Foxp3 were 474.95 ±385.51 copies of Foxp3 mRNA per 1000 copies of HPRT1 mRNA and 221.01 ±473.68 copies of Foxp3 mRNA per 1000 copies of HPRT1 mRNA for SCLC patients and for healthy volunteers, respectively. Significant differences in mRNA Foxp3 expression between SCLC PBMCs and non-cancerous PBMCs ($p < 0.001$) were disclosed. A positive Foxp3 protein nuclear fraction in 61.6% (53/86) of PBMCs from SCLC patients and in 48.6% (34/70) of control PBMC samples was assessed. The

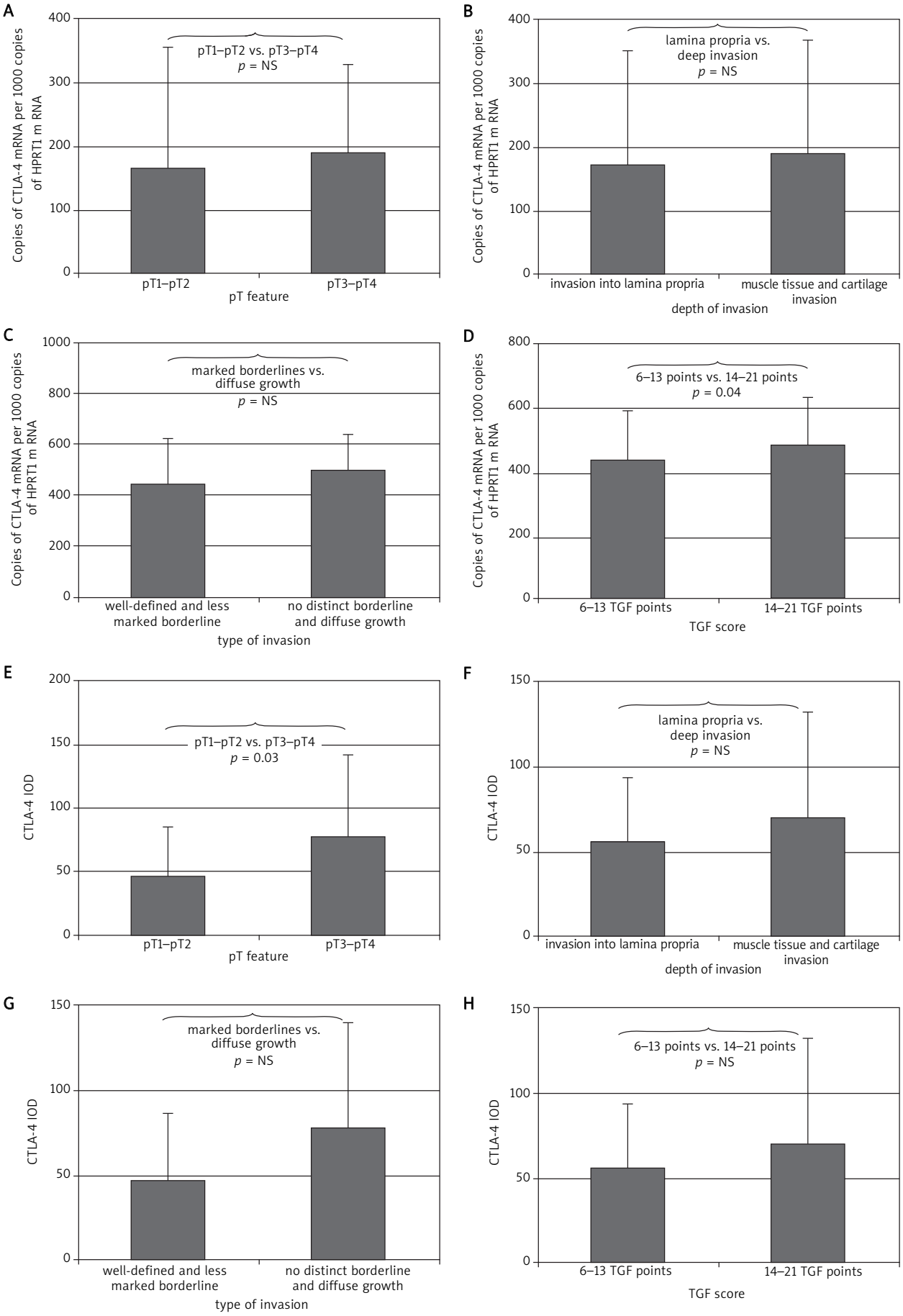

Fig. 1. CTLA-4 expression in PBMCs with regard to the clinicopathological parameters of the tumor: mRNA CTLA-4 depending on: **A**) the pT status ($p = 0.06$); **B**) the depth of invasion ($p = 0.67$); **C**) the mode of invasion ($p = 0.21$); **D**) the tumor front grading total score ($p = 0.05$); CTLA-4 protein depending on: **E**) the pT status ($p = 0.03$); **F**) the depth of invasion ($p = 0.73$); **G**) the mode of invasion ($p = 0.04$); **H**) the tumor front grading total score ($p = 0.52$)

mean value of Foxp3 protein nuclear level in both types of samples were 120.32 ±159.57$_{IOD}$ and 15.67 ±26.8$_{IOD}$, respectively. Significant differences between Foxp3 protein nuclear expression in SCLC PBMCs and control PBMCs ($p < 0.0001$) were found. A positive protein cytoplasmic fraction of CTLA-4 in 20.9% (18/86) of SCLC PBMCs was observed. In no case of the healthy donor group was a Foxp3 protein cytoplasmic fraction found. To investigate whether the mRNA status and the protein nuclear fraction of Foxp3 in the PBMCs from patients with laryngeal squamous cell carcinomas can potentially determine clinicopathological tumor features, the quantitative analysis results and Western blot outcomes were juxtaposed with the pathological assessment of the primary tumor pT and the regional lymph nodes pN status, the TFG total score as well as the type and depth of invasion. The presence of significant differences in the level of Foxp3 expression in SCLC PBMCs in relation to tumor features mentioned above was not confirmed. However, there can be seen a clear tendency to higher values for the mRNA Foxp3 in PBMCs from patients with laryngeal carcinomas characterized by more aggressive behavior determined by higher pT status, diffuse growth and indistinct tumor front borderlines with muscle tissue and cartilage invasion. Our study confirmed the presence of significant differences in the level of Foxp3 protein in SCLC PBMCs with regard to the pT status, tumor growth and type of invasion. Laryngeal carcinomas with pT3–pT4 status as well as pT1-pT2 tumors were described as SCLC with statistically different levels of Foxp3 expression in PBMCs ($p = 0.04$). In pT3-pT4 tumors the mean values of Foxp3 were lower (143.1 ±130.09$_{IOD}$) in contrast to less advanced carcinomas (75.54 ±96.39$_{IOD}$). In addition, the statistical analysis disclosed the presence of significant differences in the level of Foxp3 nuclear protein expression in PBMCs between groups of tumors with different depth of invasion ($p = 0.02$). The presence of the higher content of Foxp3 in peripheral blood cells in SCLC was more frequent for tumors with more aggressiveness determined by muscle tissue and cartilage invasion (138.95 ±130.93$_{IOD}$) in comparison tumor borders to lamina propria (62.66 ±100.5$_{IOD}$). The statistical analysis also disclosed the presence of significant differences in the level of Foxp3 expression in PBMCs according to type of invasion ($p = 0.03$). In laryngeal carcinomas characterized by diffuse growth and indistinct tumor front borderlines the mean values of Foxp3 were higher (148.38 ±107.91$_{IOD}$) in contrast to less advanced carcinomas with well-defined and less marked borderlines (75.25 ±89.8$_{IOD}$). We found that the Foxp3 protein status had no significant correlations with TFG total score. To summarize, the Western blot results obtained for SCLC with more aggressive behavior characterized by a higher pT status, more disseminated tumor invasion and deep infiltration with muscle and cartilage involvement demonstrated a higher Foxp3 expression in SCLC PBMCs. Foxp3 expression with regard to the clinicopathological parameters (pT stage, depth of invasion, mode of invasion and the tumor front grading total score) in SCLC PBMCs is shown in Fig. 2A–H. Due to the positive protein cytoplasmic fraction of Foxp3 in only a small percentage of PBMCs, statistical tests were not performed in the group studied. It should be noted, however, that all cases of SCLC with a positive level of Foxp3 cytoplasmic fraction were characterized by more aggressive cancer lesions.

Relationships between CTLA-4 and Foxp3 expression

The statistical evaluation of CTLA-4 and Foxp3 mRNA expression results did not confirm a correlation between expression of these genes. However, there can be observed a clear tendency to higher values for the mRNA Foxp3 in PBMCs derived from patients with laryngeal carcinomas characterized by higher mRNA CTLA-4 status ($r = 0.59$; $p = 0.49$). Moreover, PBMCs characterized by higher values of Foxp3 protein expression demonstrated a higher CTLA-4 protein level. Unfortunately, no significant correlations were recorded between the CTLA-4 and Foxp3 protein content. However, there can be noted an observable trend to higher values of Foxp3 expression in PBMC samples from individuals with laryngeal carcinomas characterized by higher CTLA-4 status ($r = 0.67$; $p = 0.62$).

Discussion

Regulatory T cells (Tregs) in peripheral blood and tumor infiltrating lymphocytes (TILs) play crucial roles in suppressing anti-tumor immune responses in cancer patients, and correlate with clinical outcomes [1–13, 15, 18–20, 22–30, 32–34]. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, CD152) and Foxp3 (forkhead box P3) are receptors present on Treg cells which play a critical role in the down-regulation of antigen-activated immune responses [1–34]. Many reports show that the Foxp3 and CTLA-4 expression in Tregs of patients with neoplastic disease may lead to down-regulation of CD4+ and CD8+ effector cells activation and systemic immunosuppression related to cancer progression [1–13, 15, 18–20, 22–30, 32–34]. Moreover, in the literature CTLA-4 serves as the immune checkpoint in the strategy of increasing antitumor immunity. Enhancing T-cell activation by antibody blockade of CTLA-4 provides a new approach to overcome tumor-induced immune tolerance [35].

In our study we noted positive CTLA-4 expression at both the gene and protein level in 94.2% and in 77.9% of all PBMC samples from individuals with SCLC, respectively. Positive expression of mRNA Foxp3 in 72.1% and the nuclear protein fraction level in 48.6% of all samples from patients with laryngeal carcinoma was assessed. Moreover, a clear tendency to higher average values of Foxp3 with higher CTLA-4 status in PBMCs derived from patients with SCLC was noted. Our results also indicated that the mean values of both CTLA-4 and Foxp3 were significantly higher in comparison with the healthy volunteer group results. Our findings are in agreement with the data of other researchers. According to studies reporting T regulatory cells' activity in different cancers, positive expression of CD4$^+$CD25$^+$Foxp3$^+$ Tregs in various types of neoplasms were established in 0.3–78% including the positive expression of Foxp3 in 53–91.9% of PBMC samples [2, 7, 12, 13, 18, 20, 22, 23, 25, 26, 32]. The wide range of obtained results may stem from the various fractions of Foxp3 and different suppressor phenotype of Tregs (i.e. circulating Tregs, TIL Tregs, Tr1 CD4$^+$CD25$^-$, cytoplasmic, nuclear or membranous fraction) as well as CTLA-4 (i.e. surface – surCTLA-4 and intracellular – InCTLA-4 on CD4$^+$CD25$^+$ T cells, circulating soluble CTLA4 – sCTLA4) assessed and methods used in this

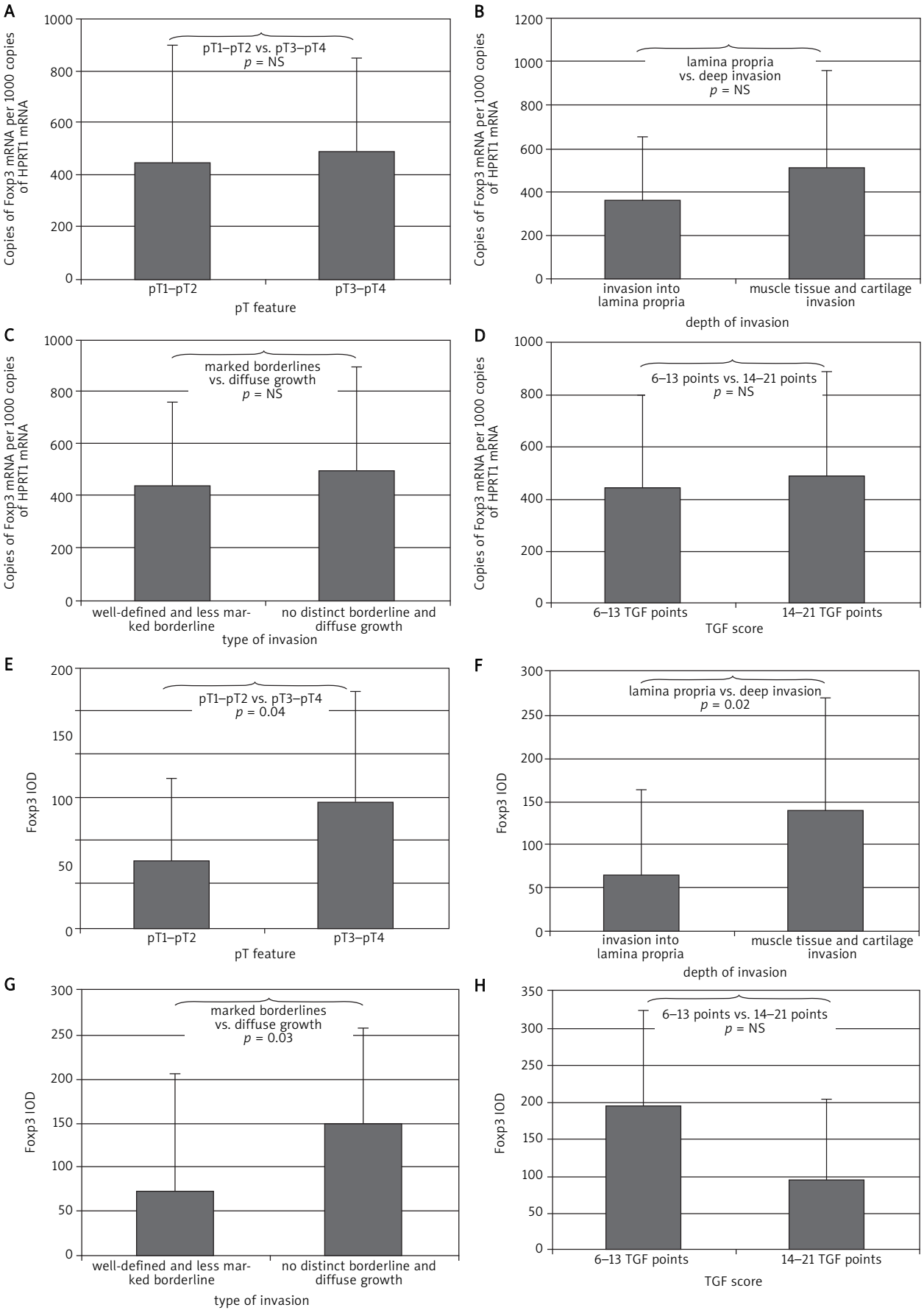

Fig. 2. Foxp3 expression in PBMCs with regard to the clinicopathological parameters of the tumor: mRNA Foxp3 depending on: **A**) the pT status (p = 0.67); **B**) the depth of invasion (p = 0.1); **C**) the mode of invasion (p = 0.49); **D**) the tumor front grading total score (p = 0.64); Foxp3 protein depending on: **E**) the pT status (p = 0.04); **F**) the depth of invasion (p = 0.02); **G**) the mode of invasion (p = 0.03); **H**) the tumor front grading total score (p = 0.38)

research as well as the different pathological grade and degree of aggressiveness of the primary tumors.

Also, in the literature a significant correlation was found between CTLA-4 and Foxp3 expressions in mononuclear cells from cancer patients [5, 7, 11, 13, 16–18, 26, 27]. Moreover, the frequency of Tregs in PBMCs was higher in individuals treated for neoplasms of various origins than that from healthy donors [9, 11, 13, 15, 17–19, 23, 33]. For example, Li *et al*. [7] noted that patients with non-small-cell lung cancer (NSCLC) had increased numbers of CD4+CD25highFoxp3+ Tregs in peripheral blood, which express high levels of CTLA-4. Also, Abo-Elenein *et al*. [9] indicated that the percentages of both CD4+CD25+ and Foxp3+ Tregs in the peripheral blood were significantly higher in breast cancer patients. Strauss *et al*. [11, 13] confirmed that CD25high clones of T cells expressed CTLA-4 and Foxp3 in PBMCs obtained from patients with head and neck cancers. In addition, suppressor phenotype and function of CD25high Treg were significantly enhanced in head and neck squamous cell carcinoma patients relative to a control group. Skewed immunological balance between Th17 (CD4+IL17A+) and Treg (CD4+CD25highFoxp3+) cells in oral squamous cell carcinoma was noted by Gaur *et al*. [15]. Moreover, Thakur *et al*. [18] reported that the expression of Foxp3 and CTLA-4 was also significantly higher in hepatocellular carcinoma cases compared to a control group. Similar results were described by Cunha *et al*. [20] for differentiated thyroid carcinomas patients.

Our research has confirmed the importance of Foxp3 and CTLA-4 gene and protein expression as indicators valuable in determining the aggressiveness of cancer of the larynx. In the SCLC group, statistically significant relationships between molecules studied and clinicomorphological parameters defined by tumor front grading criteria were demonstrated. Specifically, the average values of both CTLA-4 and Foxp3 expression in the peripheral blood T cells were higher in carcinomas with more aggressive behavior. They increased significantly either at mRNA or protein level as a higher stage of the disease, more disseminated growth (diffuse invasion or no distinct borderlines in the tumor front) and deeper neoplastic infiltration (muscle tissue and cartilage invasion) were disclosed. Unfortunately, it is difficult to find reports directly linking CTLA-4 and Foxp3 activation in peripheral blood T cells with clinicopathological features assumed to be indicators of progression of primary laryngeal carcinomas. Our study is probably the first report on the significance of both mRNA and protein fraction expression in the direct relationships with tumor front grading in carcinoma of the larynx.

The importance of Foxp3 and CTLA-4 expression in peripheral blood, neoplastic tissues and TIL infiltration was also indicated to be an unequivocal indicator of tumor progression according to clinical stage and prognosis in different carcinomas. Researchers suggest that both Treg activity and Foxp3/CTLA-4 expression play a crucial function in determination of neoplastic progression [9, 15, 17, 19–22, 24–26, 32, 33]. For example, Shen *et al*. [19] showed that the increased frequency of Treg cells in the tumor microenvironment were correlated with the cancer stage and the expression of Foxp3 mRNA increased in different TNM status in hepatocellular carcinoma. Similarly, Gaur *et al*. [15] indicated that a higher quantity of Tregs was associated with higher clinical stages and lymph node metastases in cases of oral squamous cell carcinoma. Also, Yoshii *et al*. [22] and Yuan *et al*. [24] noted a significant correlation between the expression of Foxp3 and lymph node metastases in gastric carcinoma. In the research by Cunha *et al*. [20] more intense nuclear Foxp3 staining in tumor tissue was observed in younger patients with diagnosis of differentiated thyroid carcinomas characterized by the presence of metastases. Elevated CTLA-4 expression in peripheral blood Tregs was associated with the TNM stage and metastatic or non-metastatic nodal status in lymphocytes of patients with non-small cell lung cancer in the study by Erfani *et al*. [25]. In addition, Ju *et al*. [26] reported that the percentage of CD13+CD4+ CD25high Treg cells decreased dramatically after surgical removal of tumors in patients with NSCLC. Fu *et al*. [33] demonstrated that an increased prevalence of circulating CD4+CD25highFoxp3+ Tregs was associated with high mortality and reduced survival time in hepatocellular carcinoma patients. In contrast, not all investigators have confirmed the relationship between the level of Foxp3/CTLA-4 and clinicomorphological features [17, 21, 32]. For instance, Peng *et al*. [32] found no correlation between the proportion of Treg cells and clinicopathological characteristics in hepatocellular carcinoma although the authors confirmed lower expression of Foxp3 in carcinomas with a clear tumor margin than in those with an unclear tumor margin. Zhan *et al*. [21] indicated that the presence of Foxp3 positive cells in renal carcinomas did not significantly correlate with survival and other pathological characteristics. However, the researchers demonstrated that TIL Foxp3 lymphocytes correlated with immature tumor angiogenesis. Similar results were presented by Rech *et al*. [17]. The authors did not note a difference between the absolute counts of circulating Foxp3 CD4+ T cells in early and late stage patients.

In conclusion, despite the existing differences in the final conclusions and the necessity to carry out further studies on the expression of Foxp3 and CTLA-4, these findings confirm our hypothesis regarding the role of CTLA-4 and Foxp3 in their corresponding laryngeal carcinoma behavior. Taken together, our findings contribute to a better understanding of the pathogenesis of squamous cell laryngeal carcinoma and the function of CTLA-4 and Foxp3 in this type of neoplasm. Therefore, additional research data on CTLA-4 and Foxp3 in laryngeal tumors are needed to elucidate the biological function of this molecule during tumor progression.

The authors declare no conflict of interest.

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