PD-L1 AND CD8-TUMOR-INFILTRATING LYMPHOCYTES EXPRESSION IN LARYNGEAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

Studies in recent years have shown that the immune system plays an important role in the prognosis of patients with different types of malignancies. The tumor immune microenvironment has been studied in various cancers in an attempt to find new antitumoral therapies. The therapeutic blockade of PD-1/PD-L1 has revolutionized the treatment of oncological patients, with anti-PD-L1 monoclonal antibodies having remarkable results in malignant melanoma, cervical, renal and lung cancer. In laryngeal cancer, few reports are available on the immune microenvironment and PD-L1 expression. Considering these, we retrospectively review 23 laryngeal cancer patients with laryngectomy performed in the Otorhinolaryngology Department - Emergency County Clinical Hospital St Andrew Constanta. The aim of our research was to evaluate the immunohistochemical expression of PD-L1, using combined positive score which includes both viable tumoral cells and intratumoral lymphocytes, as well as the level of CD8 intratumoral lymphocytes and to analyse the correlation between these markers and the clinicopathological characteristics of the patients, in order to set these two parameters, in the near future, as new biomarkers. PD-L1 expression was shown to be a prognostic factor for disease-free survival in laryngeal cancer patients ($p = 0.006$) and was higher in tumors with elevated CD8 lymphocyte levels, with a significant correlation between the two parameters ($p = 0.019$). The level of CD8 lymphocytes was also a predictive factor for free-disease survival, correlated with patient smoker status ($p = 0.04$), being higher in non-smoking patients.

Keywords: larynx, carcinoma, microenvironment, programmed cell death ligand-1

Introduction

It is known that malignant cells can evade immune system influence through many mechanisms, one of them being the immunosuppressive pathway of programmed cell death 1 ligand 1 (PD-L1) used by tumor cells to inhibit the antitumoral immune response. This pathway has gained great interest as it can be therapeutically targeted. Among inhibitory immune mediators, the PD-1 receptor pathway and its PD-L1 and PD-L2 ligands play an extremely important role in inducing and maintaining peripheral tolerance and T cell stability and integrity. The PD-L1/PD-L2 pathway also

potentially mediates inhibitory signals to prevent the proliferation and functioning of effective T cells with an adverse effect on antiviral and antitumor immunity (1).

Recent studies have shown that PD-L1 expression could be used for the selection of patients for anti-PD-L1 treatment (PD-1 inhibitors) and, given the success of anti-PD-1/PD-L1 monoclonal antibody therapy in several types of malignant tumors, the objective of the research described in the paper was to characterize the expression of PD-L1 and CD8 + lymphocytes (as part of the tumoral immune microclimate) in primary laryngeal carcinomas, intratumoral stromal lymphocytes being considered an

important prognostic factor in patients with head and neck squamous cell carcinomas (HNSCC), but also a prognostic biomarker for persistent/recurrent surgical laryngeal squamous cell carcinomas (SCC) (2). To date, very few studies of laryngeal carcinomas have been performed on PD-L1 and TILs expression. In 2015 and 2019, respectively, Vassilakopoulou et al (3) and Ismail et al (4) studied the correlation between PD-L1 expression and TIL stromal lymphocytes in laryngeal carcinomas. In the first study, it was selected a cohort of patients with laryngeal SCC and total laryngectomy (86.2%) or conservative procedures (13.8%), with postoperative radiotherapy in 32.7% cases and lymph node dissection in 28.5% (3).

In 2019, a meta-analysis by Jia et al (5) studied survival rates, including overall survival and disease-free survival (DFS) and free-metastasis survival on long term in patients with different levels of check-point immunological molecules. The study was based on 52 prospective papers from the literature, published between 2010 and 2018 and included oral, head and neck and pharyngeal carcinomas (7127 patients). In laryngeal carcinomas, increased PD-L1 expression is associated with better disease-free survival (DFS).

Materials and methods

The group of patients was selected from the archive of the Pathology Department, Emergency County Hospital of Constanta, from 2016-2020. Clinicopathological records were retrospectively reviewed and we collect the following data: patient age, gender, surgical procedure (biopsy, laryngectomy with or without lymphadenectomy), macroscopic tumoral and lymph nodes features and free-disease survival defined as time, in months from the date of surgery to the date of first event tumor-related. All these information were collected with approval of the Institutional Review Board. Twenty-three patients diagnosed with laryngeal SCC were eligible for the following criteria and were included in the study: treatment with curative intention, histopathological diagnosis of conventional SCC, without treatment prior to

surgery and a minimum follow-up of 12 months or until death.

Four-µm thick full sections were used for immunohistochemistry which was performed using an automated staining system (Ventana BenchMark GX Automated system, with previously validated antibodies CD8, PD-L1). For the quantification of lymphocytes, in our study the microscopic slides, for all the studied cases, were scanned at the HURON Tissuescope 4000x scanner (CEDMOG Center), both the slides in the usual HE staining and the immunohistochemistry slides for CD8. The images were then counted using the QuPath digital program, a program developed in 2017, which allows to count positive lymphocytes per mm² (6). Stromal and peritumoral lymphocytes were counted for each case, on 3 areas of 1 mm². The average of the 3 obtained values is the average number of CD8 lymphocytes / mm². For the cut-off, we calculated the average of all obtained values, thus the cut-off for CD8 lymphocytes level was 3,6.102/mm². The values from 0 to cut-off were framed at low level and from cut-off level to maximum value at high level (7).–

For a good quantification of the PD-L1 (22C3 clone) slides, it is necessary to confirm at least 100 viable tumor cells, initially confirmed on the HE slide, and the same block will be used for the immunohistochemistry sections. Positive and negative internal control slides are examined: percentage of positive cells, staining intensity and non-specific staining for both types of control. PD-L1 expression is quantified using the combined positive score (CPS), which shows the number of positive PD-L1 cells (tumor cells, lymphocytes and macrophages) divided by the total number of viable cells, multiplied by 100. Although the result may exceed 100, the maximum score is final as CPS 100. The formula for calculating CPS:

$$\text{CPS} = \frac{\text{\#positive cells}}{\text{total number of viable cells}} \times 100$$

Tumors were classified according to the system used for clone 22C3: CPS <1 (PD-L1 negative), CPS = 1-19 (PD-L1 + positive) and CPS ≥20 (PD-L1 + high level).

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS)

software program, version 26.0. Descriptive statistics were calculated, providing mean and standard deviation for continuous variables and frequency (percentage) for categorical variables. The χ^2 test was used for the comparison existing in compared groups and Spearman's correlation for correlation among variables. Kaplan– Meier analysis was performed to evaluate the survival curves and log-rank test was used to assess the statistical significance of the differences in survival. A two-tailed p-value was considered significant. The threshold of statistical significance was considered to be less than 0.05; values between 0.05 and 0.07 were considered with statistically significant intermediate value (8).

Results

In the current study, most of the cases were CD8 lymphocytes high levels (Figure 1) (15 cases, 65.21%), predominantly in patients over 60 years, all men. In patients under 60 years of age, the number of cases with high level CD8 + lymphocytes are equal to that of cases with low level (5 cases, 21.73%). Of the 15 cases of CD8 + high level, the highest percent is represented by SCC G1-G2 (11 cases, 47.82%), compared to only 4 cases (17.39%) of SCC G3. Although the statistical analysis did not show a statistically significant association, it should be noted that high CD8 + levels predominated in non-smokers (8 cases, 34.78%), exceeding the number of smokers with low CD8 + levels. Also, the number of high level CD8 cases increased in patients with SCC in the T1-T2 stage (8 cases, 34.78%), slightly exceeding that of patients with advanced stages of T3-T4 disease (7 cases, 30.43%). The low level CD8 + lymphocytes (Figure 2) were predominant in patients with SCC in the early stages of T1-T2 disease (5 cases, 21.73%).

The number of metastatic lymph nodes was lower in patients with increased CD8 + levels (9 cases, 39.13%) compared to patients with high metastatic lymph nodes (N2c-N3 stage) - only 2 cases (8.69%).

Figure 1. Squamous cell carcinoma G3 High level CD8 lymphocytes (ob.x20)

Figure 2. Squamous cell carcinoma G2. Low level CD8 lymphocytes (Ob.x20)

Of the 23 cases studied, the majority were PD-L1 negative (14 cases, 60.86%), the most positive have a lower share (9 cases, 39.13%). Of the positive ones, 5 cases (21.73%) had a PD-L1 value > 1 , and 4 cases (17.39%) had a value over 20, these after being distributed in equal range between the 2 age categories (under and over 60 years).

The degree of tumor differentiation registered differences in PD-L1 expression, thus, both well and moderately differentiated tumors (G1 and G2) and the most poorly differentiated (G3) were predominantly PD-L1 negative (Figure 3; 9 cases), 39.13% , respectively 5 cases, 21.73%. Among the poorly differentiated tumors, the percentage of positive ones is small, with only 1 case for each category (2 positive cases, 8.69%) (Table 1).

Table 1 Correlation between the main clinicopathological features of laryngeal invasive SCC, CD8 lymphocytes level and PD-L1 immunoexpression.

		CD8+ high	CD8+ low	p value	PD-L1 Negativ<1	PD-L1 Positive 1-19	PD-L1 Positive ≥20	p value (χ ² test)
Age	≤ 60	5	5	0,642	6	2	2	0,806
	>60	10	3		8	3	2	
Gender	M	15	7	0,161	1	0	0	0,715
	F	0	1		13	5	4	
Differentiation grade	G1-G2	11	5	0,809	9	4	3	0,780
	G3	4	3		5	1	1	
Smoking status	Non-smoker	8	2	0,049	5	2	2	0,653
	<40 țigări/zi	1	4		4	1	0	
	>40 țigări/zi	6	2		4	2	1	
pT	T1-T2	8	5	0,349	8	2	3	0,573
	T3-T4	7	3		6	3	1	
pN	N0-N1	9	2	0,648	5	4	2	0,323
	N2a-N2b	5	3		5	1	2	
	N2c-N3	2	2		3	1	0	
IPN	0	11	3	0,196	7	3	4	0,195
	1	4	5		7	2	0	
ILV	0	2	4	0,108	9	4	4	0,336
	1	13	4		5	1	0	
PD-L1	Negativ (<1)	6	8	0,019				
	Pozitiv (1-19)	5	0					
	Pozitiv (>20)	4	0					

Statistical analysis did not reveal statistically significant associations between PD-L1 expression and smoking-related behaviour, the number of positive and negative cases being equal in non-smokers (5 cases, 21.73%), and in heavy smokers, most cases are negative (9 cases, 39.13%), only 4 being positive (17.39%).

Figure 3. PD-L1 <1 (Negative)
Squamous cell carcinoma G2 (ob.x10)

Figure 4. PD-L1 >20 (positive) Squamous cell carcinoma
G2 (ob.x20)

The biggest percent of PD-L1 positive cases (Figure 4) were those with N0-N1 lymph node status (6 cases, 26.08%), compared with only 3 cases (13.04%) positive for N2a-N2b lymph node status and 0 case for N2c -N3. Regarding the perineural invasion, it is observed that the number of negative cases IPN0 and IPN1 are equal (7 cases, 30.43%), and the positive PD-

L1 cases did not associate perineural invasion (7 cases, 30.42%), compared to PD-L1 positive cases, with associated perineural invasion, with only 2 cases, (8.69%).

Kaplan-Meier curves and the log-rank test were performed for the analysis of disease-free survival. The level of CD8 + lymphocytes was correlated with disease-free survival, without a statistically significant association ($p = 0.98 > 0.05$), which shows that the distribution of survival is the same in the analyzed groups (Figure 5). FDS analysis correlated with PD-L1 expression showed a favourable prognosis in patients with elevated PD-L1 levels (PD-L1 > 1 and PD-L1 > 20), compared to patients with PD-L1 negative tumors (Figure 6).

Figure 5 - Kaplan-Meier DFS plot for laryngeal carcinomas categorized according to CD8 TILs level

Figure 6 - Kaplan-Meier DFS plot for laryngeal carcinomas categorized according PD-L1 expression

Discussion

To date, very few studies of laryngeal carcinomas have been performed on PD-L1 and TILs expression. In 2015 and 2019, respectively, Vassilakopoulou et al (3) and Ismail et al (4) studied the correlation between PD-L1 expression and TIL stromal lymphocytes in laryngeal carcinomas. In the first study, a cohort of patients with laryngeal SCC who underwent total laryngectomy (86.2%) or conservative procedures (13.8%) was selected, while postoperative radiotherapy was administered in 32.7%. of these, and the dissection of the lymph nodes was performed in 28.5% (3). The selected patients in our cohort with total laryngectomy and lymph node dissection, these being also eligibility criteria.

The mean age of the patients was 61.48 ± 4.42 years, a mean age lower than the 63 years of the study. The preferred location of SCC in the current study is glottic and supraglottic (70%), which overlaps with the data highlighted by the 2015 study, where it is also predominant in the proportion of 90%. Tumors in advanced stages accounted for 43%, a lower percentage compared to the mentioned study (79.6%). 14 cases (60.86%) did not show lymph node metastases, a lower percentage compared to 84.6% in Vassilokopoulou's study (3).

Regarding the level of CD8 + lymphocytes, in our study 65.21% of the cases presented high level, while in the reference study 36.1%, a much lower percentage, and the number of cases with low CD8 + level was of 63.8%, while in the current study of only 34.78%. No statistically significant correlation was observed between CD8 + level and tumor stage (T), degree of differentiation, nodal status and smoking behaviour, current results overlapping with those of 2015. The same result was obtained by Karpathiou et al., in 2016, the level of CD8 lymphocytes did not correlate with the mentioned parameters (9).

These results show that the CD3 and CD8 immune responses are a constitutive feature, independent of the clinical and histological features of laryngeal tumors, the alteration of the immune system having a real influence in immune answer (10). The proven positive prognostic role

of T cells in this study is pathophysiological correlated with the functions of these cells, a cytotoxic function by which they release granules containing perforine and granzyme (11).

In Ismail's 2019 study, PD-L1 was positive in a proportion of 92.5%, and while in the current study the percentage of PD-L1 positive was only 39.13%. In both reference studies, the level of TIL (2015) and TIL CD8+ (2019) were correlated with the expression PD-L1, a result obtained in the current study. Also, Vassilakopoulou's study showed a positive correlation between TIL and PD-L1 protein level (3). Statistically significant correlations between PD-L1 expression and lymph node status and smoking behaviour were obtained in the reference study.

In the 2015 study, TIL levels and PD-L1 expression were independent predictors of overall survival and disease-free survival in patients with laryngeal SCC, with stromal TILs levels being closely associated with a better survival. Kaplan-Meier curves showed a longer survival interval in patients with high TILs levels without being found in the current cohort of patients.

Also, a better prognosis was observed in patients with increased PD-L expression, with favorable intervals without the appearance of a new event and significantly lower risk for recurrence were highlighted by Kaplan-Meier curves and univariate cox-regression analysis.

Ismail's study showed that overall survival correlated positively with high levels of CD8+ and low levels of PD-L1 (4). There are contradictory studies regarding the role of CD8 as a prognostic factor, some highlighting the association with the prognosis (12), others refuting this association (13,14).

PD-L1 expression in tumors provides protection by reducing the activity expressed by CD4 and CD8 T cells. Anti-PD-L1 monoclonal antibodies have had remarkable results in malignant melanoma, cervical, renal and lung cancer. PD-L1 expression in head and neck cancer varies in different studies from 46.4% to 100%, with conflicting results in terms of its role as prognostic marker, as in other solid tumors (15).

These results are due to different clones, PD-L1 expression detection method, as well as different evaluation pathways and different cut-

off levels, time of tumor biopsy and tumor origin (16) or even selected sections, as PD-L1 can be expressed differently at the site of immune cell infiltration (17).

In a study that covered 305 cases of oral carcinomas, despite no observed correlation between PD-L1 expression and survival, however, when the study was limited to male smokers, the correlation existed and was negative, the prognosis being even worse the higher the PD-L1 expression (18). In another study with 55 oral carcinomas, PD-L1 expression was inversely correlated with CD8+ density, the results of the current study being similar (19), but in contrast to Karpathiou's study (9). Statistical analysis between CD8+ level and PD-L1 expression demonstrated a statistically significant association (chi-square test; χ^2 (df2) = 2; $p = 0.019$), between the two parameters showing a positive correlation (Spearman correlation $r_s = 0.516$). The meta-analysis from 2019, by Jia et al (5) studied survival rates, including overall survival and FDS in laryngeal carcinomas, increased PD-L1 expression was associated with better FDS. In our cohort, FDS average was 9 months in patients with low CD8+ level and 16 months in patients with high CD8 level, and maximum FDS was 11 and 21 months, respectively. FDS analysis correlated with PD-L1 expression, the value of $p = 0.006 < 0.05$ showing a statistically significant association between the two parameters, so it can be concluded that the distribution of survival is different in population groups. The results correlate with the most important ones by Vassilakopoulou in 2015 in terms of PD-L1 expression. Also, the results are contradictory to those obtained for other types of malignancies (kidney, colorectal, lung) (20,21), which show that an increased PD-L1 expression is associated with an unfavourable prognosis. There are studies using the 5H1 clone for PD-L1 immunohistochemical examination, in metastatic malignant melanomas, non-small lung cancer, Merkel cell carcinomas, and these have shown a positive association between high PD-L1 expression, elevated TIL and patient prognosis (22,23).

Balermipas et al. studied the immune microenvironment in head and neck cancer and showed that tumors with stromal inflammatory

infiltrate rich in cytotoxic lymphocytes CD3 and CD8 are associated with a better prognosis after chemoradiotherapy treatment (24), and the prognostic significance of tumor infiltration with CD3 and CD8 T lymphocytes has also been shown in breast, oesophageal, lung, ovarian, colon and anal cancers (25). Not only the composition of the tumor microclimate has prognostic importance, but also the location of inflammatory cells in tumors (in the vicinity of tumor islands, stroma or periphery), which may be the subject of future study in laryngeal tumors (26).

Conclusions

For the setting of PD-L1 and CD8 lymphocytes level as predictive biomarkers to help stratify patients for immunotherapeutic treatment, new studies are needed on bigger cohorts of patients, to help standardize the results, in the literature being contradictory, both on head and neck and laryngeal carcinomas. Because of the small number of patients our study has a low statistical power, but still our results can be useful for future multicentre clinical trials.

Conflict of interests

The authors declare that they have no conflict of interests.

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