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S100-positive dendritic cells in squamous cell laryngeal cancer

DANIELA EUGENIA DIACONESCU[1)], LORENA DIMA[1)], DANIELA MARIA MARINESCU[1)], MARILENA MONICA ŢÂNŢU[2)], LILIANA MARCELA ROGOZEA[1)]

[1)]*Department of Basic, Preventive and Clinical Sciences, "Transilvania" University of Brasov, Romania*
[2)]*Department of Nursing and Kinetotherapy, Faculty of Sciences, University of Pitesti, Romania*

Abstract

Dendritic cells (DC) are the most potent antigen-presenting cells, and induce antigen-specific immune responses. DC are believed to evolve into tumor-antigen pulsed cells and then to migrate to local lymph nodes, where they activate anti-tumor immune responses. This theory is supported by studies showing that high DC densities are associated with favorable prognosis in some tumor types. In the present study, we evaluated 40 primary and metastatic laryngeal carcinomas for the presence of DC, using immunohistochemistry with the anti-S100 protein antibody. We analyzed the relationship between the degree of infiltration by S100-positive (S100+) DC and prognostic factors, including histological subtype, histological grade, peritumor inflammatory infiltration, and stromal desmoplasia. The results show that in all evaluated laryngeal cancers S100-positive cells were significantly more frequent in the tumor stroma. Primary tumors with nodal metastases showed more significant differences in intraepithelial and stromal DC distribution than tumors without nodal metastases. A significant higher S100+ DC was also noticed in the desmoplasic stroma of lymph nodes. The subtype with keratinization had a significant higher S100-positive cells infiltration than the adenoid/transitional subtype. The infiltration rate of intraepithelial S100+ DC was much higher in well-differentiated (G1) tumors. No significant correlation between S100-positive cells and peritumoral inflammatory infiltration and stromal desmoplasia was found. In conclusion, dendritic cells need multiple, much more complex investigations. This work should be regarded as a preliminary investigation.

Keywords: laryngeal cancer, dendritic cells, S100 protein, prognostic factors.

Introduction

The immune system is able to detect and eliminate emerging malignant cells to prevent their uncontrolled proliferation according to the cancer immunosurveillance [1]. Dendritic/Langerhans cells (DC) play the major role in cancer immunesurveillance as the antigen-presenting cells (APC) initiating the antitumor immune responses [2].

Dendritic cells are bone-marrow-derived APC. They are considered as the most potent APC able to induce a fully primary immune responses. DC efficiently capture and process antigens. They are capable of migrating, and subsequently reaching local lymph nodes, where they activate antigen-specific T-lymphocytes. Their ability to induce strong immune responses correlates with high levels of expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules (CD80 and CD86) [3].

Infiltration of tumors by DC reflects the host immune defense mechanism. Tumor-infiltrating dendritic cells capture and process antigens shed by adjacent tumor cells and then migrate to draining lymph nodes, where they are believed to activate the anti-tumor immune responses.

There are conflicting data concerning the efficiency of DC in controlling cancer growth *in vivo*. Quantitative assessments of DC, generally identified as S100-positive DC (S100+ DC), are useful as markers for certain tumors [4].

The S100 proteins have been implicated in a variety of intracellular and extracellular functions [5]. Within cells, S100 proteins are involved in aspects of regulation of proliferation, differentiation, apoptosis, Ca^{2+} homeostasis, energy metabolism, inflammation and migration/invasion through interactions with a variety of target proteins including enzymes, cytoskeletal subunits, receptors, transcription factors and nucleic acids [6]. Several members of the S100 protein family have been correlated to prognosis in a variety of human neoplasms: lung [7], stomach [8], thyroid [9], colon [10], nasopharynx [11], larynx [12], esophagus [13], skin [14], bladder [15], breast [16], and endometrium [17]. We investigated retrospectively the density of S100-positive (S100+) DC in primary and metastatic laryngeal cancers of 40 patients, using immunohistochemistry with the anti-S100-protein antibody and performing quantitative assessments of S100+ DC. We studied the relationship between S100+ DC numbers and prognostic factors, including histological subtype, histological grade, peritumoral inflammatory infiltrate, and stromal desmoplasia.

Materials and Methods

We investigated 40 samples with primary and metastatic laryngeal cancer randomly selected from the Clinical County Hospital of Braşov, Romania, between 2009 and 2011. All tumor specimens were routinely fixed in 10% formalin, and paraffin embedded. TNM staging was performed using the Glanz system [18]. The tumor was examined microscopically to assess its histo-

logical type and grade. Grading was done according to the UICC (*Union Internationale Contre le Cancer*) system [19]. In order to certify tumoral invasion, specimens with cartilage or bone were decalcified.

Tumor-tissue nodal sections (4 μm) were deparaffinized and stained with the monoclonal anti-S100-protein antibody (Dako Corp., Denmark). Briefly, after 30 minutes incubation at 37^0C with the anti-S100-protein antibody, sections were incubated for 10 minutes at 37^0C with a secondary biotinylated antibody, then with Avidin–Peroxidase for the same time; 3,3'-diaminobenzidine was used as chromogen. Slides were then counterstained with Hematoxylin–Eosin, dehydrated and mounted. Cells were counted as DC if their structure was characteristic and if a cell nucleus could be identified; dendritic processes alone were not counted. The number of S100+ DC was assessed by counting them in the intraepithelial (IE) and stromal (ST) area of the 10 most infiltrated microscopic fields (×400) in each slide. The quantitative analysis was carried out by two observers (DD, DM).

In the present study, specimens were classified as group A+B (all laryngeal cancers), group A (primary laryngeal cancers with negative lymph node metastases), group B (primary laryngeal cancers with positive lymph node metastases), and group B-LN (matched lymph node metastases).

The mean number of intraepithelial (IE) and stromal (ST) S100+ DC was compared in all squamous laryngeal carcinomas (group A+B), and in tumors without nodal involvement (group A) *versus* tumors with nodal involvement (group B). The degree of infiltration was also assessed in the metastases of group B (B-LN). We analyzed the relationship between the degree of infiltration by S100+ DC and prognostic factors, including histological subtype, histological grade, peritumor inflammatory infiltration, and stromal desmoplasia.

Data were analyzed by Statistica for Windows 4.3 software. The Pearson's correlation was used to assess the relationship between two continuous variables. Mean differences between counts were compared with the use of *t*-tests; the calculated *p*-value less than 0.05 was considered statistical significant.

Results

The results demonstrate that S100+ cells showing typical morphology of DC, with cytoplasmic projections or veils could be found in all tumors (100%).

Figures 1 and 2 show representative examples of DC S100 immunostaining. DC were brown colored, due to 3,3'-diaminobenzidine, while other tumor cells showed a pale blue color (counterstaining with Hematoxylin). DC were mainly located between suprabasally keratinocytes.

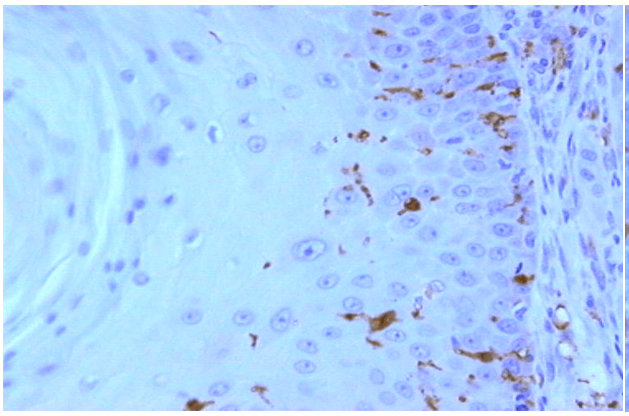

Figure 1 – ***Intraepithelial dendritic cells in a well-differentiated squamous carcinoma with keratinization. S100 immunostaining, ×200.***

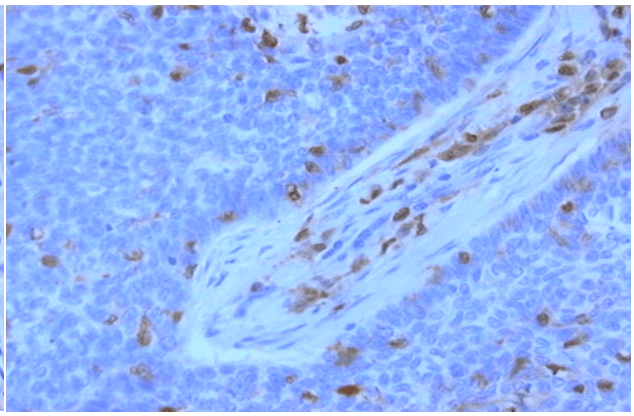

Figure 2 – ***S100+ intraepithelial and stromal dendritic cells in a basaloid squamous carcinoma. S100 immunostaining, ×200.***

Table 1 shows the mean number of S100-positive DC per 1-mm^2 tumor cells and 1-mm^2 stroma, respectively, in the different groups.

In all tumors S100-positive cells were significantly more frequent in the tumor stroma than intraepithelial (p=0.000001). Primary tumors with nodal metastases showed more significant differences in intraepithelial (p=0.000042) and stromal (p=0.0022) DC distribution than tumors without nodal metastases. A significant higher S100+ DC was also noticed in the desmoplasic stroma of lymph node metastases (p=0.005) (*t*-test for dependent samples) (Table 2).

Comparing primary tumors without nodal metastases (group A) with tumors without nodal metastases (group B), there was no significant difference of S100 intraepithelial or stromal infiltration intensity, S100-positive cells therefore having no influence on nodal involvement. As well, no difference was noticed between primary tumors (group B) and their nodal metastases (B-LN) (*t*-test for independent samples, A *versus* B and dependent samples, B *versus* B-LN) (Table 3).

Microscopic examination showed two histological subtypes of squamous laryngeal cancer: with keratinization (K) and adenoid/transitional (A/T). The subtype with keratinization had a significant higher S100-positive cells infiltration than the adenoid/transitional (p=0.0185), especially in tumors without nodal metastases (p= 0.017668) (Table 4) (*t*-test for independent samples). Unlike stromal S100-positive infiltration, the epithelial infiltration revealed no significant differences between the two histological subtypes. As well, due to the low number of cases, the basaloid subtype could not be assessed, but all the four identified cases showed lymph node metastases, suggesting that they are statistically significant, and that the S100-positive dendritic infiltration is independent of the basaloid phenotype.

The infiltration rate of intraepithelial S100+ DC is much higher in grade 1 (G1), well-differentiated tumors. There are significant differences (p=0.0364) between well-differentiated (G1) and poorly differentiated tumors (G3) (Table 5) (t-test for independent samples). Unlike intraepithelial infiltration, stromal infiltration shows no differences according to the histological grade.

Using morphometric tumor grading, the percentage of each cell type – suprabasally, spindle, and keratinocytic, was obtained. Comparing this values with the S100+ infiltration, a direct relation (r=+0,301), statistically significant (p=0.0488) with the spindle cell phenotype was found; this might explain the difference of infiltration in grade 1 and 3 tumors, in grade 3 tumor the number of suprabasally cells being higher than in the other two types (Table 6; correlations matrices).

The intensity of peritumoral inflammatory infiltration was graded I1 (low), I2 (medium), and I3 (high). No significant correlation between S100-positive cells and peritumoral inflammatory infiltration was found (Table 7; breakdown and one-way ANOVA).

The intensity of stromal desmoplasia was graded D1 (low), D2 (medium), and D3 (high). No significant correlation between S100-positive cells and desmoplasia was found (Table 8; breakdown and one-way ANOVA).

Table 1 – ***Mean number of intraepithelial (IE) and stromal (ST) S100+ dendritic cells in the different groups***

Group (A+B)[a]	Mean ± SD	No. of cases
S100 IE	348.59 ± 165.27	40
S100 ST	651.59 ± 296.54	40
Group A[b]	**Mean ± SD**	**No. of cases**
S100 IE	386.40 ± 168.23	20
S100 ST	603.39 ± 232.44	20
Group B[c]	**Mean ± SD**	**No. of cases**
S100 IE	310.77 ± 157.35	20
S100 ST	699.80 ± 348.68	20
Group B-LN[d]	**Mean ± SD**	**No. of cases**
S100 IE	330.67 ± 194.41	20
S100 ST	535.18 ± 274.15	20

[a]Group (A+B): All tumors; [b]Group A: Tumors without nodal involvement; [c]Group B: Tumors with nodal involvement; [d]Group B-LN: Metastases of group B.

Table 2 – ***Tumor intraepithelial (IE) and stromal (ST) S100+ dendritic cells***

Group	S100 IE	S100 ST	No. of cases	t-test	p-value
	Mean ± SD	*Mean ± SD*			
A+B[a]	348.59 ± 165.27	651.59 ± 296.54	40	-6.15913	0.000
A[b]	386.40 ± 168.22	603.39 ± 232.44	20	-3.54222	0.002
B[c]	310.77 ± 157.35	699.80 ± 348.68	20	-5.29177	0.00004
B-LN[d]	330.67 ± 194.42	535.18 ± 274.15	20	-3.11045	0.005

[a]Group (A+B): All tumors; [b]Group A: Tumors without nodal involvement; [c]Group B: Tumors with nodal involvement; [d]Group B-LN: Metastases of group B.

Table 3 – ***S100+ dendritic cells in the different groups***

Group	A[a] – 20 cases (Mean ± SD)	B[b] – 20 cases (Mean ± SD)	t-test	p-value
S100 IE	386.40 ± 168.22	310.77 ± 157.35	1.468321	0.150
S100 ST	603.39 ± 232.44	699.80 ± 348.68	-1.02890	0.310
Group	**B[b] – 20 cases (Mean ± SD)**	**B-LN[c] – 20 cases (Mean ± SD)**		
S100 IE	310.77± 157.35	330.67 ± 194.41	-0.364256	0.719
S100 ST	699.80 ± 348.68	535.18 ± 274.15	1.531094	0.142

[a]Group A: Tumors without nodal involvement; [b]Group B: Tumors with nodal involvement; [c]Group B-LN: Metastases of group B; IE: Intraepithelial; ST: Stromal.

Table 4 – ***S100+ dendritic cells in histological subtype K (with keratinization) versus A/T (adenoid/transitional) in the different groups***

Group (A+B)[a]	K – 23 cases	A/T – 13 cases	t-test	p-value
	Mean ± SD	Mean ± SD		
S100 ST	742.83 ± 341.06	494.26 ± 155.29	2.4749	0.018
Group A[b]	**K – 13 cases**	**A/T – 7 cases**	**t-test**	**p-value**
	Mean ± SD	**Mean ± SD**		
S100 ST	690.53 ± 239.43	441.55 ± 97.1	2.6114	0.017
Group B[c]	**K – 10 cases**	**A/T – 6 cases**	**t-test**	**p-value**
	Mean ± SD	**Mean ± SD**		
S100 ST	810.83 ± 445.89	555.77 ± 195.28	1.3133	0.210

[a]Group (A+B): All tumors; [b]Group A: Tumors without nodal involvement; [c]Group B: Tumors with nodal involvement; ST: Stromal.

Table 5 – ***S100+ dendritic cell values in grade G1 versus grade G3 tumors***

Group (A+B) (all tumors)	G1 – 8 cases (Mean ± SD)	G3 – 20 cases (Mean ± SD)	p-value
S100 IE	455.43 ± 205.27	306.83 ± 141.21	0.036
S100 ST	551.92 ± 187.85	660.52 ± 258.17	0.291

IE: Intraepithelial; ST: Stromal.

Table 6 – ***Correlation between spindle phenotype of tumor cells (S%) and intraepithelial (IE) and stromal (ST) S100+ dendritic cell infiltration***

Parameters	Mean ± SD	r	p-value	No. of cases
S%	17.32 ± 10.39			
S100 IE	348.59 ± 165.27	0.3014	0.048	40
S100 ST	651.59 ± 296.54	0.1375	0.397	40

Table 7 – ***S100-positive infiltration and peritumoral inflammatory infiltration intensity (I1, I2, and I3)***

Inflammatory infiltration	S100 intraepithelial			S100 stromal		
	Mean ± SD	*Variance*	*No. of cases*	*Mean ± SD*	*Variance*	*No. of cases*
I1	300.26± 147.78	21840.22	12	699.08 ± 251.72	63364.1	12
I2	381.35 ±179.91	32367.60	21	675.03 ±328.76	108085.7	21
I2	333.17 ±144.64	20922.69	7	499.89 ± 248.73	61867.1	7
I1 + I2 + I3	348.59 ±165.27	27316.92	40	651.59 ± 296.54	87938.4	40

Table 8 – ***S100-positive infiltration and desmoplasic reaction intensity (D1, D2, and D3)***

Desmoplasia	S100 intraepithelial			S100 stromal		
	Mean ± SD	*Variance*	*No. of cases*	*Mean ± SD*	*Variance*	*No. of cases*
D1	322.17 ± 150.53	22661.09	17	701.21 ± 340.68	116068.6	17
D2	401.26 ± 179.93	32375.47	18	627.81 ± 257.84	66483.7	18
D3	248.81 ±101.01	10203.57	5	568.49 ± 298.20	88927.8	5
D1 + D2 + D3	348.59 ±165.27	27316.92	40	651.59 ± 296.54	87938.4	40

Discussion

Dendritic cells (DC) represent a special group of cells that play a key role in inducing and maintaining the antitumor immunity, but in the tumor environment their antigen-presenting function may be lost or inefficient [20].

Infiltration of DC into primary tumor lesions has been associated with significantly prolonged patient survival and a reduced incidence of metastatic disease in patients with many types of cancers [2]. Nevertheless, other studies show that DC may present defects which make them unable to activate T-cells *in vitro* and to control tumor spread *in vivo* [21, 22]. This suggests that, in those cases, DC do not function as efficient APC in situ, and might be mediators of tumor-induced tolerance instead of immunity [23].

Dendritic cells have a particularly morphology, showing projections similar to dendrites, that enhance their contact surface and mobility [24]. In our study, by quantifying S100-positive dendritic cells, we supposed that a certain DC subpopulation, showing the mentioned morphological and immunohistochemical model, has been evaluated. Furthermore, as well as in Goldman's studies, the intraepithelial and stromal distribution made us suppose that two subpopulations of DC were quantified, differing by maturation and functional status [25].

The stromal intratumoral inflammatory infiltration is a heterogeneous population of lymphocytic cells (T-helper cells, cytotoxic T-cells, B-cells, plasma cells) and macrophages, all of them recognizing and destroying tumor cells [26, 27]. In our study, no significant correlation was identified among the density of S100+ DC and peritumoral lymphocytic inflammatory infiltration. Other authors found similar results and considered that the presence of DC and of marked lymphoid inflammation can be considered favorable prognostic factors for patients with squamous cell carcinoma of the larynx [12, 28, 29].

Doroş *et al.* considered that migration of stromal dendritic cells inside tumor areas could be an important component of the antitumor immune response and therefore, S100-positive dendritic cells may be considered as a favorable prognostic factor in laryngeal carcinomas [30]. Others concluded that DC response is not important to inhibit regional metastasis by cancer cells and therefore DC is not a reliable prognostic factor in the clinical practice [31]. However, DC may play a role in releasing an active immune response in larynx carcinomas, according to their ability to present antigens to sensitized T-cells [32].

Conclusions

Dendritic cells are the most potent antigen-presenting cells, and induce antigen-specific immune responses. In the analyzed larynx carcinomas, S100-positive DC have been observed particularly in low-grade tumors. The degree of tumor infiltration by S100 DC was not correlated with most of the analyzed prognostic factors. In squamous cell carcinoma of the larynx, most S100-positive DC were identified in the tumor stroma. This stroma contains a mixed cell population (inactive and active dendritic cells) that needs multiple, much more complex investigations. This work should be regarded as a preliminary investigation.

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Corresponding author
Lorena Dima, Lecturer, MD, PhD, Department of Basic, Preventive and Clinical Sciences, Faculty of Medicine, "Transilvania" University, 56 Nicolae Bălcescu Street, 500019 Braşov, Romania; Phone +40745–847 819, e-mail: lorenadima@yahoo.com

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