

Profile and Potential Significance of Dendritic Cells in Head and Neck Squamous Cell Carcinoma

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Abstract. *Background/Aim:* The aim of the study was the analysis of immunohistochemical expression of S100 protein and CD1a by dendritic cells (DCs) from head and neck squamous cell carcinoma (HNSCC), correlation with the histological grade, as well as analysis of the potential significance of antigen-presenting cells according to tumor location. *Patients and Methods:* Samples were collected from 50 patients with HNSCC, conventionally stained with hematoxylin and eosin for pathological diagnosis and grade, followed by immunohistochemical evaluation with S100 protein and CD1a expression. *Results:* The correlation of S100 expression in DCs with histological grading was significant ($p=0.049$). We also observed a correlation between CD1a expression and histological grading ($p=0.016$). DCs density was predominantly intratumoral for both CD1a (63% of cases) and S100 protein expression (25% of cases). *Conclusion:* Our results demonstrated the association of DCs with histological grade. Their intratumoral infiltration suggests their potential antitumor role.

Head and neck cancers (HNC) are responsible worldwide for nearly 200,000 deaths every year and are the sixth most common cancer. Head and neck squamous cell carcinoma

(HNSCC) represent around 90% of all cancer subtypes in this anatomical region. It has been shown that cancer immunity plays an important role in suppressing tumor proliferation, involving the T-cell-mediated anti-tumor immune response. During carcinogenesis, changes in the host immunological factors have been observed at several stages (1, 2).

Antigen-presenting cells (APCs) have a significant role in both innate and adaptive immunity responses, and consist of macrophages, dendritic cells (DCs) and B lymphocytes. Dendritic cells are an important component of the immune system and represent a promising approach for treating head and neck cancer, given their ability to process antigen and initiate T-cell responses. Langerhans cells (LCs) are an important subtype of DCs, known for their ability to present antigens to T lymphocytes (3). They are present in all layers of the epithelium and are most prominent in the stratum spinosum. Furthermore, it seems likely that LCs are able to induce a local cytotoxic T cell-mediated response against tumor-associated antigens, and they also appear to play a role in controlling the rate of cell division in the epithelium (4). Therefore, it has been observed that LCs are a potential factor allowing tumor progression when errors in their functionality occur.

One of the methods to identify LCs in paraffin-embedded tissues is based on the use of the antibody against S100 protein. In the case of head and neck epithelium, the limitation of the method arises because S100 proteins have been found in melanocytes from normal as well as tumor tissues. Nevertheless, LCs can be easily distinguished from other S100 positive cells based on intraepithelial distribution, association with inflammatory cells and specific dendritic morphology (5). Another method, and a more reliable identification marker for intraepithelial LCs is the immunohistochemical expression of CD1a, due to the high density of CD1a molecules in both the surface of Langerhans cells and the cytoplasm. CD1a is a transmembrane glycoprotein involved in antigen presentation of LCs (6).

Many researchers have investigated DC distribution in squamous cell carcinoma, but there has been no universal

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accepted result to date; some have observed an increase, while a few have observed a decrease in DC density, while others have reported no change (7-9). Therefore, we believe it is important to study and observe the involvement of dendritic cells in HNSCC because of their role in the prognosis and survival of these patients. Although largely investigated in patients with HNCSS, the real significance of dendritic cells remains elusive. Moreover, their roles as a prognostic marker and eventually as a target for therapy have not been explored enough to allow a definite conclusion.

To make a relevant detection of LCs, we analyzed the expression of S100 protein and CD1a by immunohistochemistry, in head and neck squamous cell carcinoma. The aim of this study was to correlate the immunohistochemical expression of S100 and CD1a and the histological grade with the clinical data. We also evaluated the density of DCs in the intratumoral and peritumoral area (10).

Patients and Methods

Patients and processing samples. The samples were collected from a total of 67 patients (only 50 patients were included in the study) from the Timisoara ENT Clinic between October 2019 and September 2020. The diagnostic and scientific study was performed in the Microscopic Morphology/Histology Department of Victor Babes University of Medicine and Pharmacy Timisoara, in collaboration with Angiogenesis Research Center Timisoara. The patient's signed consent was obtained, the principles of Declaration of Helsinki were respected, and the study was approved by the Institutional Review Board of the Scientific Research Ethics Committee of Victor Babes University of Medicine and Pharmacy Timisoara (no. 22/September 2019). The formalin-fixed paraffin-embedded tumor samples were sliced in 4 µm-thick sections, and the sections were prepared for hematoxylin and eosin staining and for immunohistochemistry detection.

Immunohistochemistry. The immunohistochemical (IHC) technique was performed with the Leica Bond-Max Autostainer (Leica Biosystems, Newcastle upon Tyne, UK). The detection of DCs was based on the anti-S100 primary antibody (polyclonal, Bond Polymer Refine Detection System, Leica Biosystems, code:PA0031) and the CD1a primary antibody (clone MTB1, RTU, Leica Biosystems, code:PA0235). The IHC technique was chosen by selecting the appropriate protocol from the autostainer software for each primary antibody. This protocol includes all the steps of the immunohistochemical technique from automated dewaxing, epitope retrieval using a high pH buffer Bond Epitope Retrieval Solution (Leica Biosystems) for 20 min, inhibition of endogenous peroxidase with hydrogen peroxide (3%) followed by incubation with the primary antibodies (30 min) and visualization with The Bond Polymer Refine Detection System (15 min). The chromogen used was 3,3-diaminobenzidine dihydrochloride and hematoxylin was used as a nuclear stain. All IHC stained samples were subsequently carefully evaluated.

Microscopic evaluation and data analysis. The samples were histopathologically analyzed and classified according to the Broder system, so the criteria for inclusion of the patients in the study was the histopathological diagnosis of squamous cell carcinoma (50

Table I. *Clinicopathological characteristics of patients and histopathological grading.*

Variables	Broder histological criteria			Total	p-Value
	G1	G2	G3		
Location					
Larynx	3	13	14	30	0.519
Oropharynx	2	4	7	13	
Nasopharynx	0	0	3	3	
Nasosinusal	0	0	2	2	
Cutaneous	0	0	2	2	
Total	5	17	28	50	
Age					
<60	1	3	9	13	0.533
>60	4	14	19	37	
Sex					
F	0	3	4	7	0.605
M	5	14	24	43	

p-Values were calculated using the Chi-squared test. F: Female; M: male.

Table II. *S100 expression correlated with Broder histological criteria.*

S100 Score	Grading			Total	p-Value
	G1	G2	G3		
0	2	11	16	29 (58%)	0.049*
+1	0	6	8	14 (28%)	
+2	1	0	2	3 (6%)	
+3	2	0	2	4 (8%)	
Total	5 (10%)	17 (34%)	28 (56%)	50	

*Significant difference. Chi-squared test.

patients). Exclusion criteria were other diagnoses as follows: lymphomas (n=6), inflammatory lesion-leukoplakia (n=5), adenocarcinoma (n=3), papillary carcinoma (n=3) and sarcoma (n=1). All HNCs were classified as well, moderate, or poorly differentiated squamous cell carcinoma. S100 protein and CD1a expression of DCs was evaluated throughout the tumor and peritumoral region by microscopic examination at high magnification (×400). The examination of sections was carried out using the Nikon Eclipse 600 photonic optic microscope and after general inspection of the sections, S100 protein and CD1a expression were evaluated as following: both were scored according to the number of cells stained per high power field (HPF) (×400) in the peritumoral and intratumoral areas of highest density, averaged over 4 fields. The score used according to DCs positive immunoreactivity: 0=negative; +1=1-20% DCs; +2=21-50% DCs; +3=51-100% DCs.

Statistical analyses. Statistical analyses were performed using MedCalc® Statistical Software version 20.015 (MedCalc Software Ltd, Ostend, Belgium; 2021). The results were statistically analyzed using Chi-squared test and a p-value of <0.05 was considered as significant.

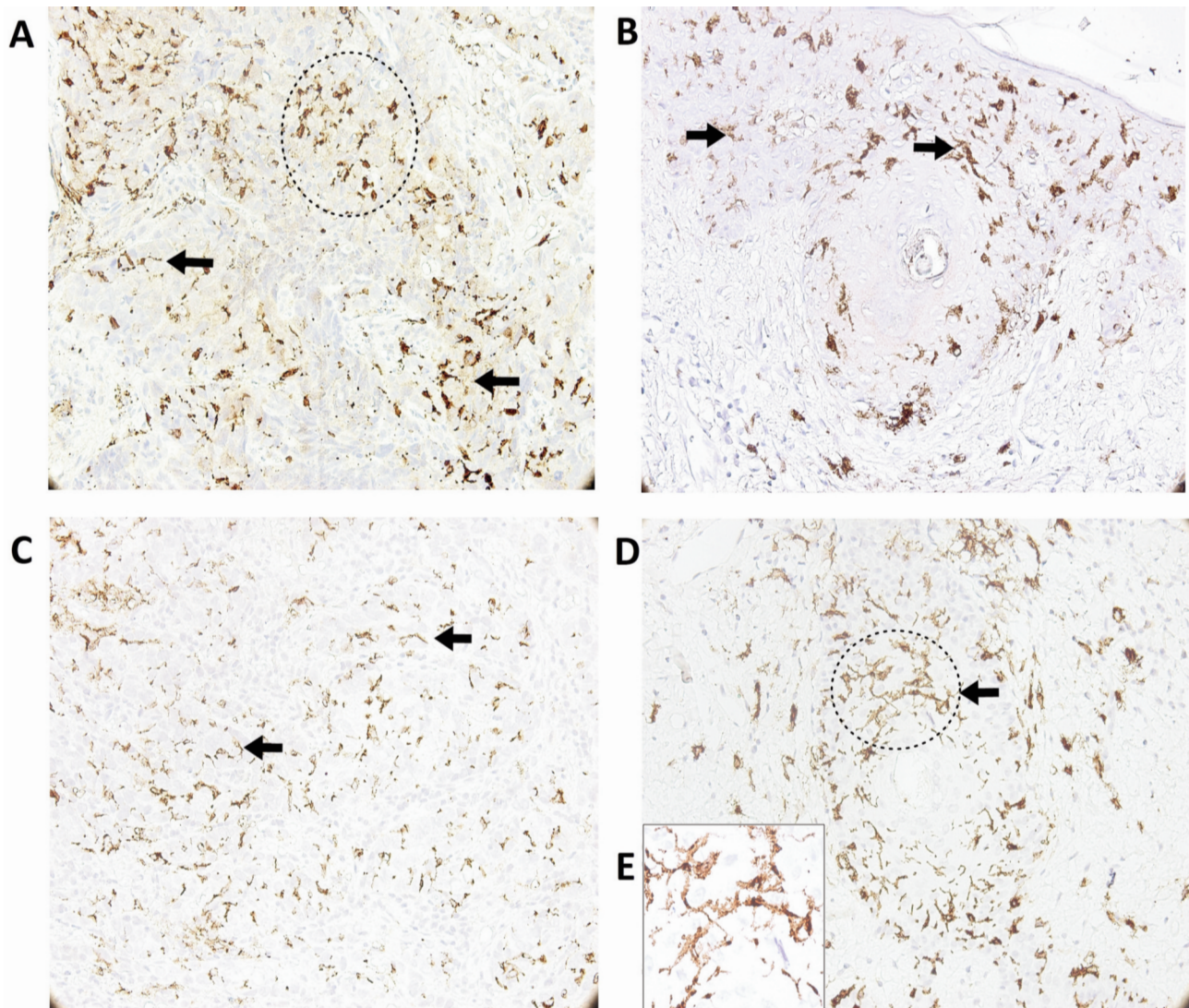


Figure 1. Immunoeexpression of S100 and CD1a, original magnification $\times 400$. Intratumoral DCs, S100 expression score = +3 (A). SSC centered by a keratin pearl surrounded by DCs, with intratumoral DCs present in basal and intermediate layer, S100 expression score = +3 (B). The DC organization is dispersed in the tumor, CD1a expression score = +3 (C). DCs around keratin pearl, CD1a expression score = +3 (D, E magnification $\times 1,000$). DCs are marked using arrows and dotted circles.

Results

The tumors were classified according to Broder histological criteria: grade G1 (well differentiated) comprised 10% of cases (n=5), grade G2 (moderately differentiated) 40% (n=20) of cases and grade G3 (weak or undifferentiated) 50% of cases (n=25). Regarding the anatomical localization, most HNSCC were present at the laryngeal level (n=30; G1=3; G2=13; G3=14), followed by oropharyngeal (n=13; G1=2; G2=4; G3=7), nasopharyngeal (n=3; G3=3), of nasosinusal localization (n=2; G3=2), and cutaneous (auricular pavilion, nasal tegument) (n=2; G3=2) (Table I).

The immunohistochemical evaluation of S100 protein expression revealed the presence of DCs in 42% of the cases, variable in number from one tumor to another. Based on DCs analysis, samples were divided into groups according to the evaluation score and correlated with histological grading (Table II). A negative or weak cytoplasmic staining of DCs was found in the basal cell compartment in most of the HNSCC samples and in the peritumoral areas. Strong DCs positive reaction was also observed in the intratumoral area, scored as +3 (Figure 1A). The correlation between S100 protein expression by DCs with histological grading was significant ($p=0.049$). A strong expression of S100 protein

was found in the intermediate and superficial epithelial cell layers of HNSCC, with a DCs score of +3 (Figure 1B). The superficial and central areas of the HNSCC specimens demonstrated a moderate and strong positive reaction with a cytoplasmic diffuse pattern of DCs, while very weak or negative expression was noticed between the tumor islands. However, S100 positive staining of DCs +1 was most common in the area of tumor invasion. Out of the total S100 positive cases, 25% of DCs were predominantly present in the intratumoral area and 17% in the peritumoral area.

The immunohistochemical evaluation of CD1a showed positive DCs in 72% of the HNSCC cases. The number of positive DCs in tumor tissue increased with decreasing histological grade (Table III). In addition, the correlation between CD1a expression and histological grading was strongly significant ($p=0.016$). Positive DCs were clearly observed in the intratumoral area in a higher percentage (63% of positive cases) (Figure 1C), compared to the peritumoral area (only 9% positive DCs). Positive dendritic cell staining was found in the basal cell layer, stem cell compartment, in most of the HNSCC samples, with a decrease in density towards the intermediate and superficial layer.

Most of the G3 differentiation grade tumors had +1 score for S100 (8 cases) and CD1 (13 cases). The tumors with keratin pearls showed a density of DCs positive for S100 protein and CD1a expression (Figure 1B and D). Furthermore, the present study did not find any association between the number of intratumoral and peritumoral DCs.

Discussion

Cancer immunotherapy has been suggested as an additional approach for HNSCC and other types of cancers. The programmed cell death 1/programmed cell death ligand 1 (PD-1/PD-L1) axis is an important target for immune therapies. HSNCC is highly infiltrated by immune cells, including tumor-infiltrating lymphocytes, T cells, plasma cells, B cells, macrophages, neutrophils, monocytes, and dendritic cells (11).

It has been shown in various studies that the presence of dendritic cells in HNSCC is associated with a better prognosis. However, due to immunosuppression, these cells do not function efficiently, which prevents the patient from stimulating an effective anti-tumor immune response and allows the tumor to continue to growing (12). The present study showed that dendritic cells are present in the tumor area of HNSCC, and a relationship with the intratumoral area for both CD1a and S100 protein expression was identified. These observations support the idea that DCs may be a promising approach for treating cancer, given the effective ability of DCs to initiate T cell responses by presenting via major histocompatibility complex (MHC) molecules (13).

The density of dendritic cells in human tumor tissues has been investigated using a variety of markers and methods,

Table III. CD1a expression correlated with Broder histological criteria.

CD1a Score	Grading			Total	p-Value
	G1	G2	G3		
0	0	8	6	14 (28%)	0.016*
+1	1	9	13	23 (46%)	
+2	1	0	3	4 (8%)	
+3	3	0	6	9 (18%)	
Total	5 (10%)	17 (34%)	28 (56%)	50	

*Significant difference. Chi-squared test.

due to their major role in the antitumoral immune response. Primarily, CD1a and S100 protein are used for investigations on tissue section, having a role in antigen presentation (14). We studied infiltration of DCs with S100 protein and CD1a expression in head and neck squamous cell tumors. CD1a positive DCs density was more significantly associated with histological grading than S100 positive cells, which showed a significant but lower bound correlation. An increased number of DCs was observed in the intratumor area for both S100 protein (25%) and CD1a (63%) expression in positive cases. The intimate relationship between tumor cells and DCs suggests the involvement of the latter in the local biology and behavior of malignancy.

Data from the literature have demonstrated the importance, involvement, and prognostic role of CD1a in highlighting DCs in HNSCC (15). In our study, we observed a strong positive response of CD1a-expressing DCs in most head and neck tumors. Also, the correlation of CD1a expression with histological classification was strongly significant ($p=0.016$), compared to the correlation of S100 expression with histological classification ($p=0.049$). At present, it is known that S100 protein has a wide distribution in human tissues (nerve cells, melanocytes, phagocytic or antigen-presenting fixed mononuclear cells, histiocytes, myoepithelial cells and various other epithelia), thus making it less sensitive in strictly detecting only dendritic cells (16, 17). Therefore, we highlight the importance of CD1a immunoexpression as a distinctive marker in the detection of DCs in head and neck tumors (18).

The literature contains little information on the comparative study of S100 protein associated to CD1a expression in HNSCC and prognosis in these patients. It has been shown that patients with nasopharyngeal carcinoma had better survival when marked DCs infiltration was present. However, there are previous studies, according to which a marked DCs infiltration of tumors is associated with an improved prognosis (19, 20). Unfortunately, given the short time since the start of the study, patient follow-up could not be performed, and it was not possible to assess patient survival. The important observation in our study is that

dendritic cell density is increased intratumorally in 63% of the cases evaluated for CD1a expression and 25% for S100 protein. We assessed the density of DCs in and around the tumor, but there was no statistical correlation when analyzing the two areas. Our results suggest that intratumoral DC density correlates with histological grading, is functionally distinct and important for antitumor immunity. In various studies it has been observed that the infiltration of dendritic cells into tumoral tissue suggests that the immune system plays an important role in tumor control (21).

Dendritic cells are important in the tumor microenvironment, whose functional changes modify the immune response. Thus, the development of targeted therapies remains a major challenge (22). There are several therapeutic strategies, including the use of antigen-presenting cells (APCs) capable of stimulating an anti-tumor immune response. So far, there have been encouraging studies using these cells as vaccines against several tumors. Vaccines using DCs in combination with another therapeutic method may be promising with significant effect in late stages of cancer, preventing recurrence or metastasis. It has been observed that the presence of dendritic cells in HNSCC is associated with a better prognosis, but due to immunosuppression (mechanism not elucidated), these cells do not function efficiently (23, 24). The perspective of further study of dendritic cells in HNSCC is a promising one on the therapeutic impact brought to the patient.

We consider that our study provides important information about DCs in head and neck squamous cell carcinoma, such as the distribution of DCs in relation to tumor area (intra- and peritumoral) and their immunorexpression for S100 and CD1a markers in different histological grades (G1, G2, G3). All this information may have an impact on the development of immune therapies in cancer and use in patient prognosis. The efficacy of a potential vaccine using DCs in HNSCC has been extensively demonstrated in tumor models. However, a pilot clinical trial has demonstrated that vaccination with DCs is a source of antigens that can provoke anti-tumor immune responses in patients with advanced HNSCC. Therefore, vaccination approaches are currently being tested in combination with other immunotherapeutic approaches and may be a future therapy used on a large scale (25). Because of the short time since the start of this study, the limitation of this research is that clinical outcomes are lacking, and we cannot assess patient survival by the Kaplan-Meier method. In conclusion, our study is original; unlike other studies that include a restricted anatomical area of study or only lymph nodes, we have obtained important data in the study of dendritic cells on several anatomical areas in HNSCC (26, 27).

Conclusion

The association of DCs with histological grading and their intratumoral infiltration suggest their antitumor role, and CD1a

expression detected by immunohistochemistry with a highly significant correlation, represents a useful marker with prognostic and therapeutic potential in head and neck squamous cell carcinoma. We conclude that immunohistochemical markers are indispensable in clinical practice, contribute to diagnosis, assess the likely course of disease and predict response to treatment. Dendritic cells are an important area of study for their involvement in cancer development. They may also represent the key to the development of immune therapies in cancer.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

CSD, design of the study and writing of the manuscript; ARC, immunohistochemistry and independent evaluation; NPG and MR, revising the text and supervision.

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