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PROGNOSTIC VALUE OF INTRATUMORAL CD57+T CELLS AND MICROVESSEL DENSITY IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

Valor prognóstico das células CD57+T e densidade dos microvasos no carcinoma de células escamosas de cabeça e pescoço

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Abstract

OBJECTIVES: This study aimed to examine the association of CD57+ T cells and MVD with clinical parameters and prognosis of HNSCC. **MATERIAL AND METHOD**: In a restrospective analysis, 43 cases of primary HNSCC have been studied. We also analysed CD57 and CD31 counting. T and N parameters was analized by Binary logistic regression analysis. Survival was analysed by Cox regression analysis. **RESULTS**: CD31 was not associated with any clinicopathological parameters. CD57 immunoexpression was associated with locorregional presence. Cox regression test showed correlation of worse survival with locorregional metastasis presence. For binary logistic parameter, WHO Grade parameter was associated with smaller tumor size and absent metastasis CD57+ T cells count was relationed with worse survival. **CONCLUSION**: There was no association between MVD and clinicopathological parameters. Locorregional metastasis presenting high CD57 positivity. No association was found between CD57 and the other clinicopathological parameters. Multivariate analysis showed that individuals presenting locorregional metastasis were associated with poor survival. CD57 count and WHO grade were associated with larger tumor size.

Keywords: Squamous cell carcinoma. Head and neck. Risk factors. CD57 protein. CD31 protein. Angiogenesis.

Resumo

***OBJETIVO**: Estudou-se a associação de células CD57+T e densidade microvascular com parâmetros clínicos e prognóstico do carcinoma de células escamosas de cabeça e pescoço (CCECP). **MATERIAL E MÉTODO**: Em análise retrospectiva, 43 casos de CCEP foram estudados. Analisamos também a contagem de CD57 e CD32. Os parâmetros T e N foram analisados pela análise da regressão logística binária. A sobrevivência foi submetida a anális de regressão de Cox. **RESULTADOS**: CD31 não foi associada com nenhum parâmetro clinicopatológico. A imunoexpressão do CD57 associou-se com presença locorregional. O teste de regressão de Cox demonstrou correlação entre pior sobrevivência com presença locorregional de metástase. Para o parâmetro de logística binária, o parâmetro da OMS foi associado com tumores menores e ausência de metástases. A contagem das células CD57+ foi relacionada com a pior sobrevivência. **CONCLUSÃO**: Não houve associação entre microdensidade vascular e parâmetros clínico-patológicos. Metástases locorregionais apresentaram alta positividade para CD57. Não foi encontrada associação entre CD57 e outros parâmetros clínico-patológicos. Análise multivariada demonstrou que indivíduos apresentando metástases locorregionais apresentaram pobre sobrevida. Contagem de CD57 e grau da OMS foram associados com tumores maiores.*

***Palavras-chave**: Carcinomas de células escamosas. Cabeça e pescoço. Fatores de risco. Proteína CD57. Proteína CD31. Angiogênese.*

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is a disease associated with major morbidity and mortality and represents a worldwide public health problem in many countries (1, 2). HNSCC arises as consequence of multiple molecular events influenced by effects of various carcinogenic habits, like tobacco and alcohol use. Other environmental factors in a background of inherited resistance or susceptibility (3). Genetic influences in HNSCC have been found in cases of squamous cell carcinomas but the their components are not entirely clear (4, 5). The interaction of tumor development and the host immune system, as well as the impact of the immune system on the tumor clinicopathological parameters is not completely defined yet. Besides, it was thought that patients whose tumors had high number of tumor infiltrating inflammatory cells would have a good prognosis, while patients with few or no tumor infiltrating inflammatory cells would have a poor prognosis (6).

Blood supply is essential for tumor growth and metastasis, since the blood not only supplies nutrients and oxygen but it also provides a vascular route for hematogenous spread to distant sites (3). Although these processes are very complex, several studies have suggested that measuring the microvessel density (MVD) of tumors may be of prognostic value (4). CD31 is a molecule of endothelial cell adhesion and it is sensible in the endothelial cells investigation and is used on analisis of MVD in normal, displasic and neoplasic cells (2).

CD57+ T cells are important source of cytokines and interact with other immune cells to trigger an adaptive, or antigen-specific, immune response. CD57+ T cell subset has the ability to directly kill target cells, as well as mediate antibody-dependent cell killing. It is also able to eliminate malignant tumor cells without recognizing tumor-specific antigen or prior sensitization, characterizing them as important effector cells in control of diseases and treatment of human cancer (7-11) (Figure 1).

The prognosis of HNSCC is usually poor, which may be related to a greater impairment of cellular immunity and angiogenesis in head and neck cancer than other malignancies. The correlation between these processes and the clinical course of HNSCC and prognosis has not been adequately substantiated.

The aim of this study was to examine the association between CD57+ T cells and MVD with clinical parameters and prognosis of the disease in patients with primary HNSCC. We also correlated survival time of these patients with T and N parameters.

PATIENTS AND METHODS

The study group was a series of 43 fully reviewed cases of primary HNSCC which were treated between 1998 and 2006, at public services of head and neck surgery in Montes Claros - Minas Gerais State, Brazil. No patient who received preoperative radiotherapy or chemotherapy was selected for this study. Medical records of these patients were retrieved. The term young is used to define patients with 45 years or younger at time of diagnosis, whereas old is defined as patients older than 45 years at time of diagnosis. Ethical approval for this study was obtained from the local ethics committees (Unimontes/COEP, 167/2005).

Clinical Staging and Anatomical Site

All patients were staged according to the UICC TNM Classification of Malignant Tumors (1997) (6). HNSCC was classified according to the primary site as described in the International Classification of Diseases (ICD-10) for Oncology (WHO, 1990).

Histopathological study

Histological sections of all samples were stained with hematoxylin-eosin and histopathologically evaluated under conventional light microscopy. All malignant tumor samples of head and neck were classified as squamous cell carcinoma. Histopathological classification of the tumors as well, moderately, or poorly differentiated was based on the World Health Organization criteria (7, 8). The microscopic features of invasive front grading of HNSCC were analyzed according to classification of Bryne et al. (9, 10). This system evaluates tumor cell morphology and tumor-stroma interaction as determined by degree of keratinization, nuclear polymorphism, pattern of invasion and lymphocytic infiltration. Each morphological feature was scored from 1 to 4 points, yielding a total malignancy score. A poorly differentiated score was considered to be suggestive of a tumor high malignancy. Tissue sections were analyzed by one independent observer, who were unaware of the patients' cancer familial history or disease staging.

Immunohistochemical Method

The primary antibody against CD57 (1:100, Mouse Monoclonal Anti-CD57, Clone NK-1, Zymed Laboratories, Inc., San Francisco, CA, USA) and CD31 (1:100, Mouse Monoclonal Anti-CD31, Clone PECAM-1 Novocastra Lab., New Castle, UK) were detected by the LSAB kit (DAKO, Denmark). Negative controls were performed by replacing the primary antibody with phosphate-buffered saline.

A sample of lymph node with intense staining for CD57 was included with each immunostaining procedure and considered as positive control. Only cells that presented membrane and/or cytoplasm with brown-colored staining in lymphocytes were considered positive. The number of CD57+ T cells was evaluated in the region of invasive front of HNSCC. All cells were enumerated in 20 representative and consecutive microscopic high-power fields (X400) and, at this magnification, each field (integration graticule) had an area of 0.096 mm^2. Areas of normal mucosa, scar tissue, salivary glands, muscle, necrosis and lymph node were excluded. The total area studied in each tumor was then of 1.922 mm^2. The mean count of CD57+ T cells refers to the number of positive cells CD57+ T cells in infiltrating tumor stroma, and including those presented in the tumor parenchyma. Only lymphocites cells that presented a membranous brown-colored staining were considered positive. CD57 was measured as the percentage of immunostained lymphocites cells.

A sample of hemangioma with intense staining for CD31 was included with each staining procedure and served as positive control. The intensity of staining was not considered to CD31, only enough to distinguish positive from negative cells. The method described by Weidner et al. (11, 12) was employed for this study. The area with the greatest density of CD31 positive endothelial cells was designated as the "hot spot". Counting was performed on five separate high power fields (X 400, integration graticule with area of 0.096 mm^2) within this hot spot. All stained endothelial cells or cell clusters were counted as one microvessel. The presence of a lumen was not required. Microvessel density (MVD) was defined as the sum of vessels found in three hot spots (Figure 1).

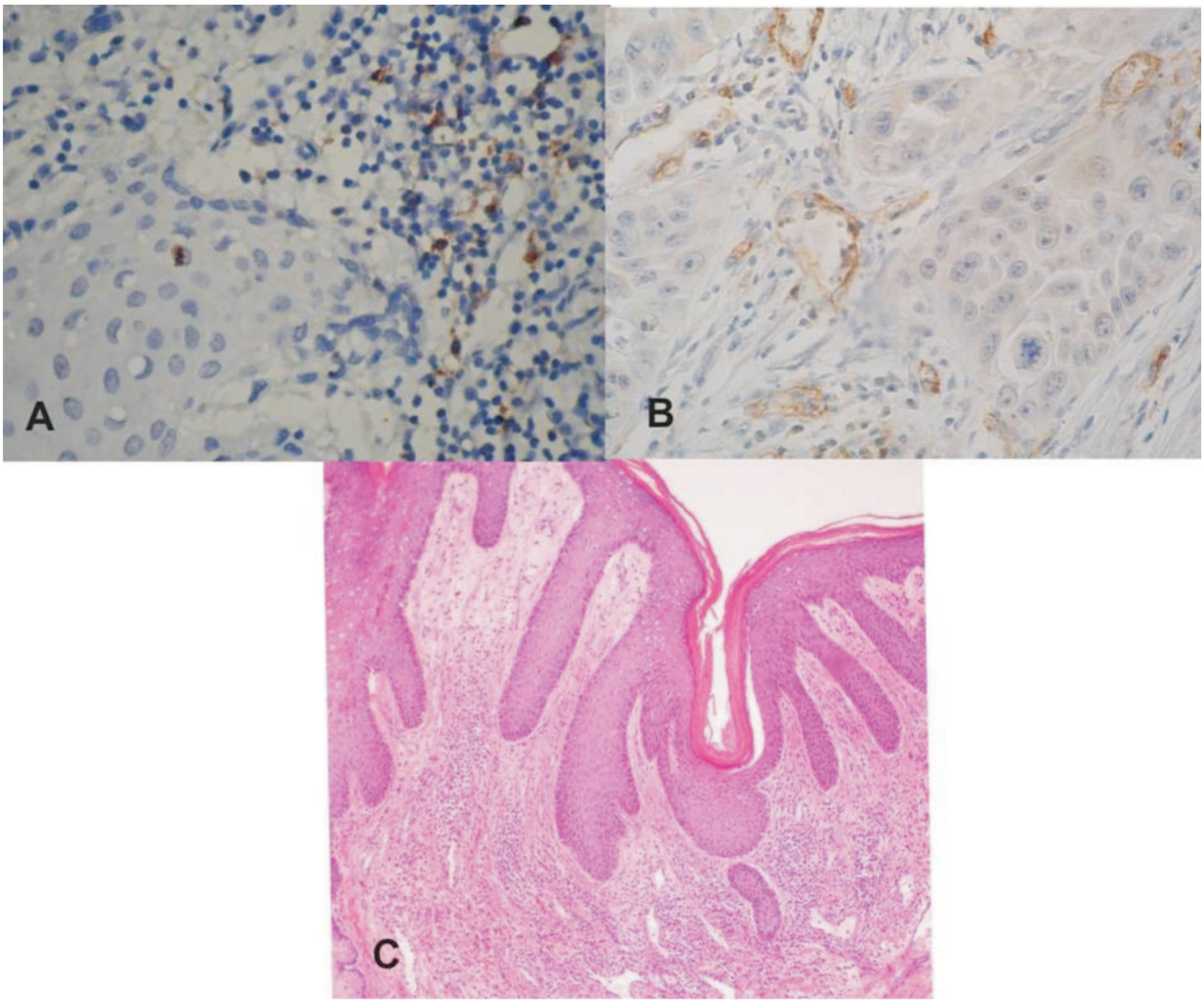

Figure 1 - Expression of CD57 (**A**) and CD31 (**B**) in head and neck squamous cell carcinoma. (Staining: DAB. Counterstained: Mayer's hematoxylin. A and B, magnification 400 x). Representative picture of head and neck squamous cell carcinoma (**C**). (Staining H&E, C, magnification 100 x)

Statistical analysis

The possible associations of epidemiological and clinicopathological parameters with CD57 e CD31 count were examined by χ^2 test with the Fisher exact test. To this analysis, CD57 and CD31 were categorized according to median. Statistical analysis showing a confidence above 95% ($p < 0.05$) was considered significant. The results of Kaplan-Meier were compared by the log-rank test. Age and variables with $p \leq 0.25$ were included in the Cox proportional hazards regression to estimate predictive factors of crude survival. The model was adjusted to the best significance. The factors examined were those recorded at the time of diagnosis. The records of each patient were reviewed considering the same parameters since 13 to 2447 days (including censured patients). Odds ratios (ORs) for complaint associated with tumor development and their respective 95% confidence intervals (CIs) were estimated using binary logistic regression analysis. Logistic regression analysis was used to build a model of variables which could result a better explanation to the risk of presenting an advanced tumor. In Kaplan-Meier, Cox regression and binary logistic regression tests, it was considered the CD57 and CD31 counts. All statistical analyses were

performed with the statistical pack SPSS® (SPSS Inc., Chicago, IL, USA), version 17.0 for Windows®.

RESULTS

The male-to-female ratio was 7.6:1. Table 1 shows the distribution of the HNSCC cases according to the clinicoepidemiological status. Fourteen (32.56%) patients were less than 45 years. The age was categorized into young (≤ 45 years) and old (> 45 years) (Table 1). Table 2 shows the comparative analysis of the clinicoepidemiological data and risk factors with immunohistochemical results. Locoregional metastasis occurrence was associated with high CD57+T cells (p = 0.033).

Table 1 - Distribution of HNSCC samples according to epidemiological states

VARIABLES	All n (%)
Gender	
Female	5 (11,6)
Male	38 (88,4)
Ethnicity	
White	18 (41,9)
Non White	25 (58,2)
Cancer Family History	
Absent	19 (44,2)
Present	24 (55,8)
T parameter	
T1/T2	11 (25,6)
T3/T4	32 (74,4)
N parameter	
N0	15 (34,9)
N1.N2.N3	28 (65,1)
TNM	
I/II	4 (9,3)
III/IV	39 (90,7)
Smoker Grade	
No tabacco habit	2 (4,6)
Low	10 (23,3)
Moderately	23 (53,5)
Heavy	2 (4,7)
Ex tabagista	6 (14,0)
Drinker Grade	
No alcohol habit	3 (7,0)
Low	6 (14,0)
Moderately	3 (7,0)
Heavy	15 (34,9)
Ex	16 (37,2)
Invasive Front Grade	
I	3 (7,0)
II	20 (46,5)
III	20 (46,5)
III	20 (46,5)

Table 2 - Distribution and analysis of epidemiological, risk factors and clinical parameters in the HNSCC, according to MVD and CD57+ T Cell expression

	MVD			CD57+ T cell		
	Low	High	p value	Low	High	p value
Age						
Young	9	5	0,324	8	6	0,586
Old	14	15		14	15	
T parameter						
T1/T2	5	6	0,536	8	3	0,097
T3/T4	18	14		14	18	
N parameter						
N0	10	5	0,205	11	4	0,033*
N1.N2.N3	13	15		11	17	
TNM Clinical Staging						
I/II	2	2	0,883	3	1	0,317
III/IV	21	18		19	20	
Invasive Front Grade						
F1	2	1		2	1	
F2	12	8	0,568	10	10	0,856
F3	19	11		10	10	
WHO Grade						
I	7	8		10	5	
II	6	5	0,787	4	7	0,283
III	10	7		8	9	
Drinker Grade						
No alcohol habit	0	3		3	0	
Low	1	5		3	3	
Moderately	1	2	0,039*	1	2	0,497
Heavy	11	4		7	8	
Ex	10	6		8	8	
Anatomic site						
Bucal cavity	8	10		9	9	
Oropharinx	5	7	0,121	7	5	0,700
Larinx	8	1		5	4	
Hipofarinx	2	2		1	3	

All values were calculated by χ^2-statistical test.
* Results statistically significant.

Curves illustrating the rate of survival according to T and N parameters are shown in Figure 2.

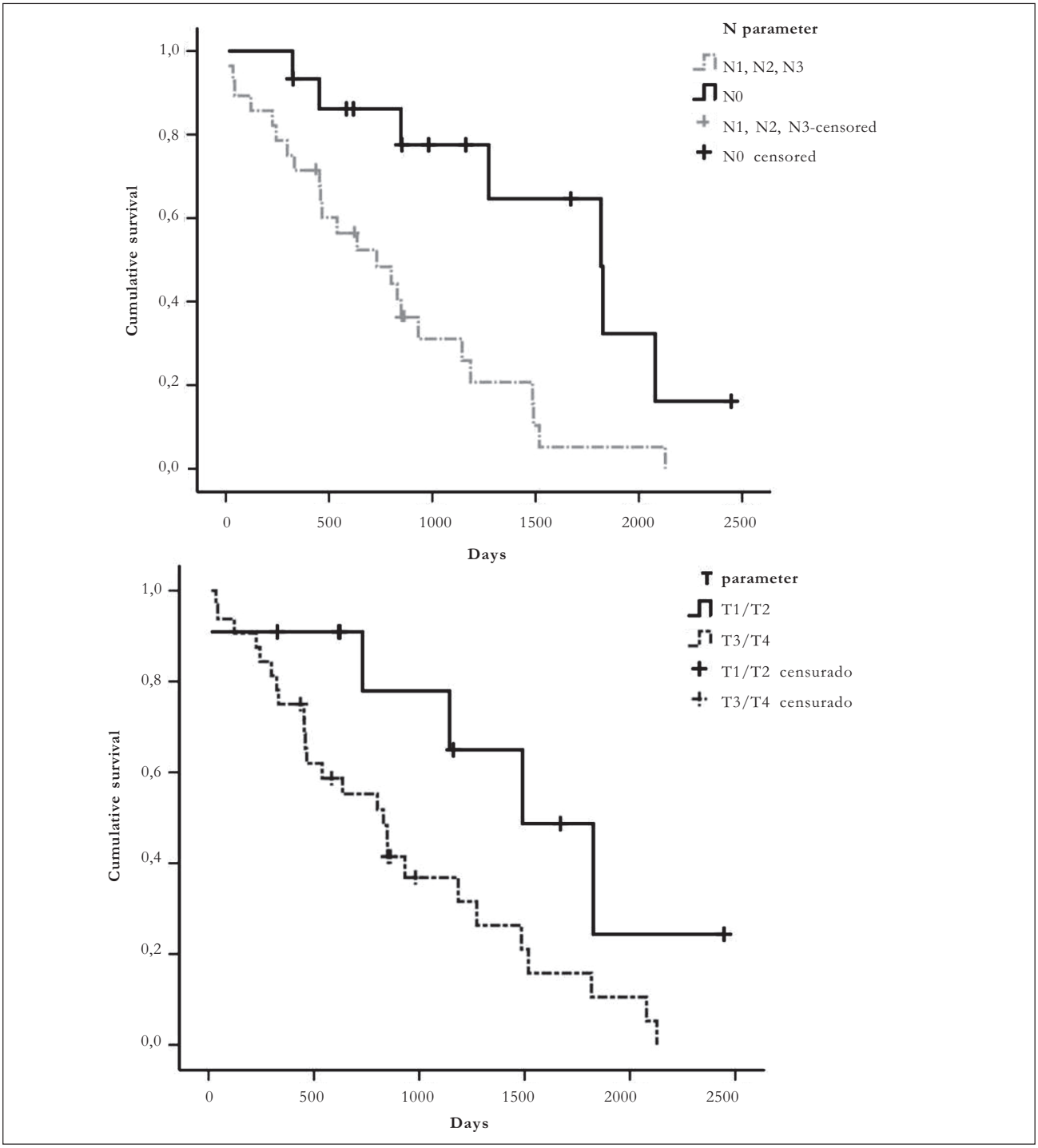

Figure 2 - Survival in patients with HNSCC according to N parameter (A) and T parameter (B) days, respectively. Difference between the two groups was evaluated by log-rank test and the differences were statistically significant (p = 0.003 and p = 0.039, respectively)

The cox regression analysis showed that N parameter (OR = 9.647; p < 0.007) was associated with poor survival (Table 3).

Table 3 - Distribution and analysis of epidemiological, risk factors and clinical parameters in the HNSCC, according to MVD/CD57+ T Cell expression

	T cell CD57+/ MVD				
	High T cell CD57+/High MVD	**High T cell CD57+/Low MVD**	**Low T cell CD57+/Low MVD**	**Low T cell CD57+/High MVD**	**p value**
Age					
Young	4	2	5	3	0.702
Old	8	7	6	8	
T parameter					
T1/T2	1	2	4	4	0.352
T3/T4	11	7	7	7	
N parameter					
N0	1	3	9	2	0.001*
N1.N2.N3	11	6	2	9	
TNM Clinical Staging					
I/II	0	1	2	1	0.514
III/IV	12	8	9	10	
Invasive Front Grade					
F1	1	0	1	1	
F2	5	5	7	3	0.650
F3	6	4	3	7	
WHO Grade					
I	3	2	4	6	
II	4	3	2	2	0.741
III	5	4	5	3	
Drinker Grade					
No alcohol habit	6	2	4	7	
Low	0	3	1	2	0.046*
Moderately	0	2	1	0	
Heavy	6	2	5	2	
Anatomic site					
Bucal cavity	5	4	3	6	0.253
Oropharinx	3	2	2	5	
Larinx	3	1	5	0	
Hipofarinx	1	2	1	0	

All values were calculated by χ^2-statistical test.
* Results statistically significant.

For binary logistic parameter, WHO Grade (OR = 40.446; p = 0.039) parameter was associated with smaller tumor size and absent metastasis (OR = 12,818; p = 0.049). CD57 (OR 1,239; p = 0,052) parameter was associated with larger tumor size (Table 4).

Table 4 - Cox regression analyzes in the HNSCC patients with follow-up of 2447 days with censored cases

VARIABLES	OR	Lower	Upper	p value
CD57 Count	1.018	0.989	1.048	0.231
N parameter				
N0		**Reference**		
N1.N2.N3	9.647	1.850	50.321	0.007*
WHO Grade				
I		**Reference**		
II	0.598	0.176	2.038	0.412
III	1.455	0.355	5.960	0.602

OR: odds ratio; CI: condence interval; n: total number.
The model was fitted to the best significant model

DISCUSSION

HNSCC has been characterized as a model neoplasm in which prognosis is mainly determined by the clinical stage (8, 10, 13). Smoking and alcohol drinking habit are very common in Latin America, specially in Brazil, and are frequently associated with unfavorable socio-economical condition. The carcinogenic action might be stronger or not depending on the anatomical site (14-16). The analysis of our results showed that majority of patients with HNSCC had considerable history of tabacco and alcohol consumption.

Angiogenesis is the process where new blood capillaries arise from pre-existing vessels. Endothelial cells are the main cellular group in angiogenesis process. They are stimulated to form vessels by pro-angiogenic factors. Angiogenesis is critically important to vascular system formation during human development and for the growth of solid tumors (17, 18). Increased microvessel density has been correlated with disease progression and poor prognosis in HNSCC. The vasculature of HNSCC is often disordered as a consequence of overproduction of factors promoting vessel formation (19). Besides, the degree of angiogenic activity in cancers is variable (20-22). In our study, MVD was not associated with any clinicopathological parameter.

CD57+ T cells presence in cancer has an important role in host defense mechanism. The expression of appropriate adhesion molecules has been showed for rapid and efficient migration of CD57+ T cells from the peripheral blood to tumoral stroma. Evidences suggest that characteristics of tumor cells may induce CD57+ T cells mediate antitumor reactivity. Consequently, not only the presence but the CD57+ T cells activities have also been studied in tumor microenviroment of HNSCC (23-26).

In our study, an association was demontrated between CD57+ T cells and N and T parameters. The activities of CD57+ T cells have been reported in patients with poor prognosis. Some studies reported possible mechanisms for the lack of control of tumor growth by CD57+ T cells (25, 27-29).

Metastasis presence was associated with worse survival by Kaplan-Meier test. Furthermore, N and T parameters were the predictors of survival in HNSCC patients. Survival trends for HNSCC have shown little change during the last few decades in worldwide. Late diagnoses has also been reported in other studies as predictors of survival in HNSCC (30-32). Among HNSCC Brazilian patients, the survival rate is frequently poor. Sociodemographic and cultural factors, patient education and awareness issues, and/or delays in diagnosis have been pointed as important factors associated with poor prognostic (33). It is complex for patients to identify lesions in difficult sites for perception by themselves. The adequate care by professionals would result in better performances of treatments in patients with HNSCC.

CONCLUSION

In conclusion, there was no association between MVD and parameters clinicopathological parameters. The current study we found patients

with locorregional metastasis presenting high CD57 positivity, suggesting this protein as an important marker for identification of individuals of this risk group. No association was found between CD57 and the other clinicopathological parameters. Multivariate analysis showed that individuals presenting locorregional metastasis were associated with poor survival. CD57 count and WHO grade were associated with larger tumor size.

Considering the methodological limitations of this study, it is essential the contribution of multicentric investigations for an improvement on knowledge about genetical events and their clinical applicability. Our study adds further support to the scientific process of identification and prognostic analyses of complaints.

CONFLICT OF INTEREST STATEMENT

The authors declared no conflict of interest in the present manuscript.

INFORMED CONSENT STATEMENT

The patients signed an informed consent, kept in the records, in the archives of the UNIMONTES University.

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