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ORIGINAL ARTICLE

# Evaluation of CD57+ cells in oral squamous cells carcinoma and their relationship with clinicopathological parameters

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## Abstract:

**Introduction:** The immune response occurring in the tumor microenvironment is very complex. Surface molecule CD57 can be expressed by natural killer cells and/or activated T lymphocytes, which are key cellular types in the cancer immunity. **Objectives:** This study investigated CD57 immunoexpression in immune cells of oral squamous cell carcinoma (OSCC) and verified its association with clinicopathological features. **Methods:** Paraffin-embedded sections of 45 OSCCs were submitted to morphological and immunohistochemical analysis using anti-CD57 antibody. Relationship between the expression of CD57 and clinicopathological parameters (age, metastasis to regional lymph nodes, clinical stage and histopathological grade of malignancy) was verified by Pearson's Chi square, and Fisher exact statistical tests. **Results:** The majority (64.4%) of the OSCCs analyzed affected subjects of male gender and over 40 years old (68.9%). There was a significant association between histological grade of malignancy and presence of lymph node metastasis, as well as clinical stage ($p<0.05$). Conclusions: The results suggest that CD57+ cells immune cells infiltration is a consistent finding in OSCC, regardless of clinicopathological features of these tumors.

**Keywords:** Mouth Neoplasms; Antigens, CD57; Immunohistochemistry.

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## INTRODUCTION

Several studies showed that inflammatory infiltrate characteristics play an important role in the oral squamous cells carcinoma (OSCC) biological behavior[1-3]. In 1970, Burnet[4] described that the host immune system is responsible for surveillance, recognition and destruction of transformed cell clones before giving rise to tumors, in addition to possess the ability to destroy tumors already formed. Currently, it is known that immune components in tumor microenvironment may prevent cancer progression, but can also promote tumor growth[1,5]. Therefore, many studies have been focusing on the participation of the immune system in the tumor microenvironment, aiming to discover new prognostic factors and therapeutic targets.

The main types of immune cells defending the host against cancer are cytotoxic cells, such as natural killer (NK) cells in innate immunity and $CD8^+$ cytotoxic lymphocytes in the acquired immunity[6]. Surface molecule CD57, also known as HNK-1 or Leu-7, is a glycoprotein present in NK cells going through terminal differentiation[7], therefore many authors have been using CD57 as a marker for NK cells[1,5]. However, CD57 is not restricted to NK cells, since it is also expressed by activated T cells, including $CD4^+CD57^+$ and $CD8^+CD57^+$ T cells, which lack proliferative capacity, but are able to secrete cytokines[8,9].

CD57 protein has been used to investigate the functional immune response in patients with cancer and other diseases with significant involvement in the inflammatory component, such as auto-immune diseases[7]. In tumor biology, $CD57^+$ cells infiltration is associated to better prognosis in several types of cancer[10,11], including OSCC[1,12].

In this light, this study aimed to investigate the immunohistochemical expression of CD57 in OSCCs microenvironment; and evaluate if this expression shows association with clinical and histopathological parameters.

## METHODS

### *Tissue specimens and Clinical data*

This study was approved by an institutional ethics committee (Protocol No. 266.863/2013), and consists of a retrospective analysis of 45 OSCCs stored tissue blocks diagnosed between 2002 and 2012 in two oral pathology services. The sample included all cases with specimens that had sufficient amount of biological material for morphological and immunohistochemical analysis. All cases of squamous cells carcinoma (SCC) of the lip and base of tongue, as well as cases previously treated with chemotherapy or radiotherapy were excluded. Data regarding gender, age, ethnicity, habits, location of lesions, presence or absence of lymph node metastasis and clinical stage according to the tumor–node-metastasis (TNM) system were obtained from the medical records.

### *Morphological study*

Paraffin sections (5 µm) were routinely prepared and stained with Hematoxylin & Eosin for examination by light microscopy. Histopathological grading of malignancy was performed as proposed by Bryne[13]. Four parameters were analyzed in the tumor invasive front: degree of keratinization, cellular pleomorphism, invasion pattern, and inflammatory infiltrate intensity. Score of 0 to 4 was attributed to each parameter and cases with a total score $\leq 8$ were classified as low-grade and those with score $> 8$ were classified as high-grade malignancy as performed by Silveira et al.[14].

### *Immunohistochemistry*

Formalin-fixed paraffin-embedded tissues were cut into 3-µm thick sections and mounted on organosilane-coated slides (3-aminopropyltriethoxy-silane; Sigma Chemical Co., St. Louis, MO, USA). The material was submitted to immunohistochemistry technique by the streptavidin–biotin method (LSAB, Labeled Streptavidin Biotin) using a monoclonal antibody anti-CD57 (clone 8144B; Dako North America, Inc., Carpinteria, CA, USA) with 1:100 dilution, antigen retrieval in TRIS/EDTA solution (pH 9.0) for 3 minutes in Pascal and incubation of 60 minutes. The reaction was developed with diaminobenzidine (DAB) as chromogen and the specimens were counterstained with Mayer's hematoxylin and mounted in Erv-resin (Easy Path®). Healthy cervical lymph node specimen was used as a positive control, while replacement of primary antibody with bovine serum albumin was used for negative control.

### *Analysis of immunostained cells*

All slides were scanned for immunostaining analysis (Pannoramic MIDI, 1.15 SPI, 3D HISTECH®, Budapest, Hungary) and Pannoramic Viewer 1.15.2 software (3DHISTECH®, Budapest, Hungary) was used to examine the whole specimen. Tumor invasion front areas with greater immunoreactivity were chosen. From the chosen areas, photographs of 5 consecutive fields

(100μm) were taken and exported to Imaging Processing and Analysis in Java software (ImageJ®, National Institute of Mental Health, Bethesda, Maryland, USA) for cell counting.

Cells exhibiting brown staining in the cytoplasm or plasma membrane were defined as positive, regardless of the staining intensity. After the sum of the values obtained in each field, the mean positive cells for each case was calculated. The median value of the mean $CD57^+$ cells were used as parameter to classify the cases as presenting low expression (mean of positive cells < median value) and high expression (mean of positive cells > median value).

### *Statistical analysis*

Data originally presented in Excel® format (Microsoft® Office XP Professional) were exported to Statistical Package for the Social Sciences software (version 20.0; SPSS, Inc., Chicago, IL) for statistical analysis. Pearson's Chi-square or Fisher exact test were used to evaluate the association between CD57 expression in OSCC and the clinicopathological variables, as well as between clinical and morphological data. All tests were two-sided, and *p*-values of less than 0.05 were considered statistically significant.

## RESULTS

Forty-five patients with OSCC were intentionally selected to this research. Table 1 shows the demographic, clinical and morphological data. Twenty-nine individuals (64.4%) were male and 16 (35.6%) were female. The mean age was 53.36±16.75 years, ranging from 23 to 79. Thirty-one patients (68.9%) were aged over 40, while only 14 (31.1%) were aged equal or less than 40 years. With regard to skin type, 26 patients exhibited dark brown/black skin (57.8%), while 19 (42.2%) were light-skinned. Almost all OSCCs (n = 41; 91.1%) composing the present sample affected the tongue. Most patients had no habits related to alcohol consumption or smoking (42.2%), while 11 reported synergistic use of tobacco and alcohol (24.4%).

Regarding clinical stage (TNM), seventeen (37.8%) patients were classified as stage III, followed by stage II (35.6%). Metastasis in cervical lymph nodes was detected in 37.8% of cases and 28 (62.2%) were classified as high-grade of malignancy, whereas 17 (37.8%) as low-grade. Table 2 shows that the OSCCs studied demonstrated a highly significant association ($p<0.001$) between clinical variables (metastasis in cervical lymph

**Table 1.** Sample profile according to demographic, clinical and histological features.

| Variable | n (%) |
|---|---|
| Gender | |
| Male | 29 (64.4) |
| Female | 16 (35.6) |
| Age | |
| ≥ 40 years | 32 (71.1) |
| < 40 years | 13 (28.9) |
| Type of skin | |
| Light | 19 (42.2) |
| Dark Brown/Black | 26 (57.8) |
| Habits* | |
| None | 19 (50.2) |
| Alcohol consumption | 1 (2.6) |
| Tabagism | 7 (18.4) |
| Alcohol consumption + Tabagism | 11 (28.9) |
| Location | |
| Tongue | 41 (91.1) |
| Gingiva | 2 (4.4) |
| Palate | 1 (2.2) |
| Floor of the mouth | 1 (2.2) |
| Clinical Stage (TNM) | |
| Stage I | 9 (20.0) |
| Stage II | 16 (35.6) |
| Stage III | 17 (37.8) |
| Stage IV | 3 (6.7) |
| Metastasis to lymph nodes | |
| Yes | 17 (37.8) |
| No | 28 (62.2) |
| Histological grade of malignancy | |
| Low grade | 17 (37.8) |
| High grade | 28 (62.2) |

*In seven cases, this information was unavailable.

**Table 2.** Absolute and relative distribution of high and low grade of malignancy OSCCs according to metastasis to lymph nodes and clinical stage.

| Parameters | Histological grade of malignancy | | TOTAL n (%) | $p^{(1)}$ |
|---|---|---|---|---|
| | High grade n (%) | Low grade n (%) | | |
| Metastasis to lymph nodes | | | | |
| Absence | 11 (39.3) | 17 (60.7) | 28 (100.0) | <0,001* |
| Presence | 17 (100.0) | 0 (0) | 17 (100.0) | |
| Clinical Stage (TNM) | | | | |
| Stages I and II | 9 (37.5) | 15 (62.5) | 24 (100.0) | <0,001* |
| Stages III and IV | 19 (90.5) | 2 (9) | 21 (100.0) | |

(1) Pearson's Chi-square test. * Results are statistically significant.

nodes and clinical stage) and histopathological grade of malignancy. All cases exhibiting metastasis in cervical lymph nodes showed a high grade of malignancy and most of cases with advanced clinical stages were high-grade tumors (n=19; 90.5%).

CD57 immunostaining was observed predominantly in the membrane and sometimes, in the cytoplasm of mononuclear rounded shape inflammatory cells present in the stroma at invasion front of all OSCC analyzed. A high CD57 expression (Figure 1) was observed in 23 (51.1%) cases and a low expression (Figure 2) was present in 22 (48.9%) cases. No significant association was observed between the clinical (age, metastasis to lymph nodes and clinical stage) variables and CD57 immunoexpression, neither between this expression and histological grade of malignancy (Table 3).

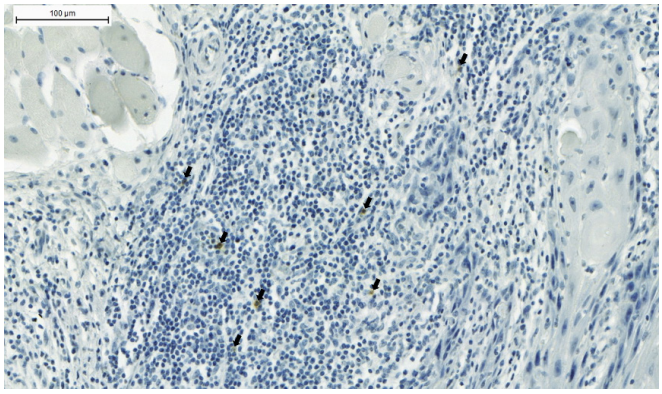


**Figure 1.** Photomicrograph showing CD57 expression (arrows) at invasive front of high-grade of malignancy OSCC (LSAB; Bar indicates 100 µm).

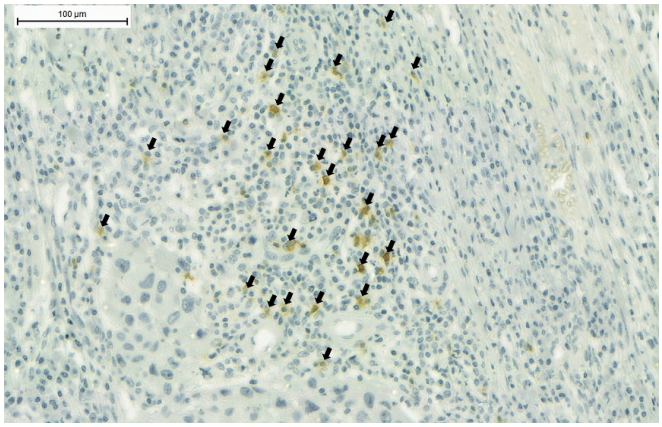


**Figure 2.** Photomicrograph showing CD57 expression (arrows) at invasive front of low-grade of malignancy OSCC (LSAB; Bar indicates 100 µm).

**Table 3.** Absolute and relative distribution of CD57 immunoexpression in OSCCs according to clinicopathological variables.

| Variables | CD57 immunoexpression | | TOTAL n (%) | $p^{(1)}$ |
|---|---|---|---|---|
| | Low expression n (%) | High expression n (%) | | |
| Age | | | | |
| ≥ 40 years | 18 (56.2) | 14 (43.8) | 32 (100) | 0.121 |
| < 40 years | 4 (30.8) | 9 (69.2) | 13 (100) | |
| Metastasis to lymph nodes | | | | |
| Absence | 12 (42.9) | 16 (57.1) | 28 (100) | 0.299 |
| Presence | 10 (58.8) | 7 (41.2) | 17 (100) | |
| Clinical Stage (TNM) | | | | |
| Stages I- II | 11 (45.8) | 13 (54.2) | 24 (100) | 0.661 |
| Stages III-IV | 11 (52.4) | 10 (47.8) | 21 (100) | |
| Histological grade of malignancy | | | | |
| Low grade | 6 (35.3) | 11 (64.7) | 17 (100) | 0.155 |
| High grade | 16 (57.1) | 12 (42.9) | 28 (100) | |

(1) Pearson's Chi-square test

## DISCUSSION

Squamous cell carcinoma is the most common malignant neoplasm of the head and neck region, including oral cavity. Despite advances in treatment, when located in this area, it shows poor prognosis, with 5-year survival rates in less than 40% of cases, representing a major public health issue in the world[15,16]. Etiology of OSCC is complex and multifactorial, since it can result as a combination of several intrinsic (e.g. genetic, nutritional deficiencies and immunosuppression) and extrinsic factors such as tobacco smoking, alcohol consumption and viruses, especially Human Papilloma Virus (HPV)[17].

Typically, OSCC affects mostly the tongue of men over 60 years old, although epidemiological studies have shown an increased incidence in young adults in recent years[16]. There is controversy in the literature regarding the etiology, natural history and prognosis of OSCC affecting younger patients, given that these individuals are not often exposed to typical extrinsic risk factors such as smoking and alcohol consuming[16,18]. These differences suggest a divergent biology of young-onset OSCC. The demographic characteristics of the present sample, as well as tumor location, agree with those reported in the literature[1,2,19]. However, most of the individuals reported to have no harmful health habits, such as smoking or alcohol consumption, regardless of age.

Currently, important indicators of OSCC aggressiveness include clinical staging, presence of metastases in cervical lymph nodes and some morphological parameters and/or histopathological grading[20]. OSCC metastasis to cervical lymph nodes occurs in about 33%-45% of cases[1,15,21], similar to the rate found in our study. We also observed that patients clinical staging and lymph node metastasis status showed a significant association with histopathological grading, corroborating previous studies[22].

Even though traditional classification tools, such as TNM system, provide important source for patient management, it is well known that clinical course can significantly vary among patients with same tumor stage. Therefore, a comprehensive analysis of the tumor immune components and their interactions with tumor cells can portray significant impact in the cancer clinical course, response to treatment and may complement the current tumor classifications.

This is particularly interesting for oral cancer, given that oral tumors microenvironment is rich in immune cells and soluble factors induced and released by these cells[23]. Moreover, knowing that aging is accompanied by changes in many functions of human immune system[24], one can wonder if the immune reaction occurring in the tumor tissues may show a divergent immune context between young and old patients with OSCC. In this context, we investigated the relationship of OSCC clinicopathological parameters with $CD57^+$ cells, which include NK cells.

NK cells constitute an early defense system against foreign and autologous cells suffering from stress (e.g. microbial infection, malignant transformation and physical or chemical injuries), representing the first line of defense against tumors[6]. In solid tumors, high density of NK usually correlates with better prognosis, therefore therapeutic strategies to increase the amount of these cells may improve current immunotherapy protocols[10,11].

$CD57^+$ NK cells are considered as cells in terminal differentiation, comprising cells with high cytotoxic potential, but decreased sensitivity to cytokines and reduced replication capacity[7]. Therefore, the acquisition of CD57 follows the natural differentiation of NK cells, in which $CD57^+$ NK cells are a stable population of mature cells, which increase according to aging and pathogens exposition[2,6,7].

Tumor-infiltrating T cells directed against tumor-associated antigens are indicative of a spontaneous intratumoral immune response in cancer patients[25-27]. Differentiation of $CD57^+$ T cells within the tumor microenvironment is thought to result from chronic stimulation by tumor antigens[28]. These cells are considered 'senescent-like' T-cells, although they are not functionally exhausted, since they maintain the capacity to secrete cytokines (e.g. TNF-α and IFN-γ) when exposed to with cognate antigen[7,8]. Evidences suggest that accumulation of $CD57^+CD8^+$ T cells is a frequent event in different types of cancer, such as renal cell carcinoma[29] and gastric cancer[10], while $CD57^+CD4^+$ T cells has been linked to a variety of haemato-oncological diseases[30].

Intratumoral evaluations show that a low density of $CD57^+$ inflammatory cell is associated to poor prognosis in patients with OSCCs. Zancope et al.[5] investigated the CD57 expression in inflammatory cells of oral potentially malignant lesions, OSCCs and SCCs of the lower lip, which usually have a less aggressive clinical course than the intraoral cancer. Their results showed a higher CD57 expression in SCCs of lower lip compared with the other two lesions.

Taghavi et al.[12] showed that CD57 expression was an independent prognostic factor for OSCC, as the high level of $CD57^+$ NK cells correlated with longer overall survival for their sample. Türkseven and Oygür[1] demonstrated that intratumoral density of $CD57^+$ cells were lower in OSCCs presenting poor prognosis. On the other hand, increase in the proportion of peripheral populations of $CD57^+CD4^+$ and $CD57^+CD8^+$ T-cells were significantly associated to advanced clinical stage, and therefore may act as effector cells for OSCC progression, exerting influence the systemic immunity of patients with OSCC[19].

In our sample there were no significant associations between CD57-positive immune cells and clinicopathological factors, corroborating the results of Taghavi et al.[12] Fraga et al.[2] showed an association between high $CD57^+$ cell density and development of large sized head and neck SCCs and presence of locoregional metastatic disease and suggested that peritumoral infiltration of $CD57^+$ inflammatory cells represent senescent-like cells that could be associated with a type of local immunosuppression that might support cancer growth and locoregional metastasis.

Our results can therefore suggest that $CD57^+$ immune cells infiltration is a consistent finding in OSCC immune contexture, regardless of clinicopathological variables, which may reflect the cytotoxic activity of $CD57^+$ NK cells and chronic stimulation of $CD4^+$ or $CD8^+$ T cells subpopulations by tumor antigens, supporting an immunoregulatory role for these cells.

## CONCLUSION

Histopathological grading of malignancy was associated with the clinical status of the present sample; therefore, reflecting the tumors biological behavior, at least to some extent. The inability to perform phenotypic profiles to differentiate between T or NK cells was the major limitation of the present study. We demonstrated that OSCC's microenvironment is highly infiltrated by $CD57^+$ immune cells, regardless of patients age, tumor stage, or morphological grading. Future investigation focusing on specific subsets of $CD57^+$ cells can add valuable information about the impact of these inflammatory cells in OSCC microenvironment.


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None.


### Conflicts of interest

The authors declare that they have no conflict of interest.

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