Cross-sectional Study

PAI-1 expression in intratumoral inflammatory infiltrate contributes to lymph node metastasis in oral cancer: A cross-sectional study

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ABSTRACT

Introduction: Immune cells contribute with mediators in the protein expression profile of the tumor microenvironment. Levels of plasminogen activator inhibitor-1 (PAI-1) are elevated in non-malignant inflammatory conditions; however, the association between PAI-1 expression and inflammation remains uncertain in oral squamous cell carcinoma (OSCC). This study aimed to investigate PAI-1 expression in mononuclear inflammatory cell infiltrate in OSCC and its role as a prognostic marker.

Methods: Samples were collected from patients with OSCC, treated surgically, and followed for 24 months after the procedure. Thirty-nine tumoral tissue were analyzed using immunohistochemistry. Correlation between protein expression, clinicopathological parameters, and the prognosis was investigated.

Results: Positive PAI-1 expression in mononuclear inflammatory cell infiltrate was significantly associated with lymph node status (p = 0.009) and with the cytoplasmic expression of vascular endothelial growth factor A (VEGFA) (p = 0.028). Multivariate analysis revealed weak PAI-1 expression as an independent marker for lymph node metastases, with approximately 8-fold increased risk compared to strong expression (OR = 8.60; CI = 1.54–48.08; p = 0.014).

Conclusion: Our results suggest that the strong PAI-1 expression in intratumoral inflammatory infiltrate is an indicator of a better prognosis for patients diagnosed with oral squamous cell carcinoma.

1. Introduction

Head and neck cancer (HNC) are the seven most incident cancers in the world. In 2018, according to Global Cancer Statistics, the HNC caused 450,000 deaths [1,2]. The main risks for HNC are alcohol drinking and tobacco smoking affecting primarily men between 50 and 70 years of age [3].

Oral squamous cell carcinoma (OSCC) is the most frequent type of HNC, and yearly, it contributes 389,000 for new cases [4]. OSCC is characterized by poor prognoses and low survival rates, with a percentage survival of about 50% in five years, and the main therapeutics procedures include surgery, radiotherapy, and chemotherapy [3]. The epidemiology disease is complex due to multifactorial and multigenic characteristics; it is linked with individual genetic susceptibility factors, lifestyle choice, and environmental agents that subjects are daily exposed [5,6].

The immune cells act as mediators of the tumor microenvironment and promote differential protein expression in tumoral tissue, resultant...
in an inflammatory environment. The inflammation contributes to tumorigenesis through activation of protein pathways that promote angiogenesis and modify the extracellular matrix leading to metastasis and tumor progression [7,8]. In OSCC, cellular immunity presents deficits related to tumor progression, including changes in monocyte chemotaxis and defects in the interaction between antigen presenting monocytes and lymphocytes [9].

The occurrence of metastasis has a relation with fibrinolysis. The main step to this process occurs by the fibrin degradation mediated by plasmin. The fibrinolytic system is formed by urokinase cellular receptor (uPAR), urokinase (uPA), and plasminogen activator inhibitor-1 (PAI-1), and this system shows the relation with clinical aggressiveness of tumors and with the regulation of local inflammatory responses [10-12].

In the fibrinolytic system, the urokinase plasminogen activator (uPA) binds to its receptor (uPAR) and after this ligand, zymogen is converted into plasmin, which can degenerate extracellular matrix components like laminin, fibronectin, and collagen. Previous research suggests that uPA/uPAR is an important proteolytic system in cell invasion. In contrast, PAI-1 acts to inhibit the uPA/uPAR complex, which allows the binding of the low-density lipoprotein receptor-related protein 1 (LRP-1) at this complex, promoting the internalization and lysosomal degradation of uPA/PAI-1, and recycling of uPAR/LRP1 [12-14].

The metastatic process also involves the activation of several intracellular pathways. These include the vascular endothelial growth factor A (VEGFA) stands out because it promotes morphogenesis of the blood vessels, by vasculogenesis or angiogenesis. Neangiogenesis promotes the supply of nutrients and oxygen to the tumor cells and enables the migration and establishment of these cells in other tissues [15,16].

Furthermore, PAI-1 and VEGFA are synthesized from the action of the hypoxia-inducible factor-1 transcriptional complex (HIF-1). This molecular pathway promotes the expression of genes that prevent cell death under hypoxia conditions [17].

The correlation between prognosis and PAI-1 expression in mononuclear inflammatory cell infiltrate has only recently been explored. Based on this information and on the importance of the microenvironment in tumor progression and prognosis, the present study aims to analyze the relation between PAI-1 expression in the intratumoral inflammatory infiltrate, clinicopathological features, and prognosis in oral squamous cell carcinoma.

2. Materials and methods

2.1. Ethics

This study was approved by the Medical and Health Research Committee of the Heliopolis Hospital on September 13th, 2011 (CEP n° 818), São Paulo, Brazil. Written informed consent was obtained from all patients participants in this study, that followed the Declaration of Helsinki [18].

2.2. Samples

All samples from this study were collected by the Head and Neck Genome Project (GENCAPO), São Paulo, Brazil. Therefore, 39 tumoral tissue samples were used for immunohistochemical analysis of PAI-1 in mononuclear inflammatory cell infiltrate. The patients with OSCC were surgically treated at the Head and Neck Surgery Department of Heliopolis Hospital, between January 2002 and December 2008, and were followed for 24 months after surgery. The exclusions criteria used in the research were lip carcinomas, previous treatments, non-removal of cervical lymph nodes, distant metastasis, and positive surgical margins. To confirm the diagnosis and select appropriate areas for immunohistochemical analysis, histopathological slides were reviewed by a senior pathologist. All the tumors were classified following the TNM system (8th edition) [19].

2.3. Immunohistochemistry

The sections of tumor tissue with 3-μm thick were subjected to the process of dewaxing with xylene and rehydration with graded ethanol. Then, the sections were treated with 10 mM citrate buffer solution, pH 6.0, and heated for 20 min in a microwave oven for retrieve antigenicity. After cooling off for 15 min at room temperature the endogenous peroxidase activity was blocked by incubation of slides in 3% hydrogen peroxide for 20 min. After washing in tris buffer solution, pH 7.4, tissue sections were incubated with protein blocking (Spring Bioscience) for 15 min to block non-specific staining. Then, the sections were washed with tris buffer solution and incubated with monoclonal antibodies anti-PAI1 antibody mouse 1: 100 (abcam®, Cambridge, MA, EUA, cat # 125687) and anti-VEGFA antibody mouse 1: 100 (abcam®, Cambridge, MA, EUA, cat #1316), at 4 °C overnight. The reaction was amplified with a REVEAL Polymer-HRP, mouse/rabbit (Spring Bioscience), according to the manufacturer’s protocol, and was visualized with a 3,3 ‘-diaminobenzidine as the chromogen. All sections were then counterstained with hematoxylin. Positive controls for PAI-1 (human hepatocellular carcinoma) and VEGFA (human hemangiomma) and negative controls (absence of primary antibody) were used (supplementary material Fig. 1A, B, C, and D).

The semiquantitative analysis was performed following the methodology adapted from Santos et al. [20], Peterle et al. [21], Maia et al. [22] and considering the percentage of cells stained at 0 when 0% of labeled cells, 1 when 1-25% of the cells were positive; 2 when 25-50% of the cells were positive; and 3 when > 50% of the cells were positive; and by staining intensity in negative (0), weak (1), moderate (2) and strong (3). Scores received from percentage and staining intensity were multiplied and their means calculated for each sample and the resulting score was used to categorize PAI-1 in the intratumoral lymphocytic inflammatory infiltrate expression as strong (≥3) or weak (<3). VEGFA cytoplasmic tumor expression and PAI-1 cytoplasmic and membrane tumor expressions were categorized as negative (0 and 1) or positive expression (≥1). All analyses were performed two different analyzers independently, and conflicting cases were re-analyzed. This study has been reported in line with the REMARK criteria [23].

2.4. Statistical analysis

Chi-square and Fisher Exact tests were used, and the values were confirmed using the Lilliefors test (significance was p<0.05). Multivariate Logistic Regression was used to adjust the Odds Ratio value (OR) and the Confidence Interval (95% CI). To calculate the survival rate, the day of surgery was considered the starting point, and the endpoint was considered the date when the disease recurrence or when death occurs. For alive patients, the date of the last return appointment was used as the endpoint. The designs of the Disease-Free Survival, Local Disease-Free Survival, and Disease-Specific Survival curves were obtained using the Kaplan-Meier model and adopting the Wilcoxon p-value. Hazard Ratio (HR) and 95% CI values were adjusted using the Cox Proportional Hazards multivariate model. Statistical calculations were performed using Epi-Info® v3.4.3, 2007, and SPSS® 19.0 software.

3. Results

Among the individuals analyzed, the mean age was 68.0 ± 11.4 years, 12 (30.8%) being men, and 27 (69.2%) women. With regards to the anatomical location of tumors, 15 (38.5%) were on the tongue, 12 (30.8%) were on the floor of the mouth, 10 (25.6%) were on the gum, 2 (5.1%) were in the retromolar area. The tumor descriptions and their clinical and pathological characteristics are reported in Table 1.

PAI-1 expression in mononuclear inflammatory cell infiltrate was weak in 14 (35.9%) samples, and strong positive in 25 (64.1%) (Fig. 1A, B and C). PAI-1 expression did not show a significant association with tumor characteristics such as size (p = 0.475), differentiation grade (p =
Table 1
Epidemiological features.

| Features          | Cases |
|-------------------|-------|
|                   | No    | (%)  |
| Gender            |       |      |
| Female            | 27    | (69.2) |
| Male              | 12    | (30.8) |
| Age, years        | 68.0  |      |
|                  | (±11.4) |      |
| Smoking           |       |      |
| No                | 24    | (61.5) |
| Yes               | 15    | (38.5) |
| Alcohol consumption |     |      |
| No                | 25    | (64.1) |
| Yes               | 14    | (35.9) |
| Total             | 39    | (100.0) |

Fig. 1A. Immunohistochemistry (IHC). Strong PAI-1 in lymphoid cells of intratumoral inflammatory infiltrate. Strong positive expression. The magnification was 400×. The scale bar indicates 10 μm.

Fig. 1B. Immunohistochemistry (IHC). Weak PAI-1 in lymphoid cells of intratumoral inflammatory infiltrate. Weak positive expression. The magnification was 400×. The scale bar indicates 10 μm.

0.303), lymphatic invasion (p = 0.754), perineural invasion (p = 0.099), PAI-1 cytoplasmic tumor expression (p = 0.075), and PAI-1 membrane tumor expression (p = 0.281) (Fig. 2A and B), but the expression was significantly associated with lymph node status and VEGFA cytoplasmic tumor expression (p = 0.009; p = 0.028, respectively) (Table 2). The VEGFA cytoplasmic expression in oral squamous cell carcinoma was negative in 17 (43.6%) samples and positive in 21 (53.8%) (Fig. 2C and D, Table 2).

Multivariate analysis showed that weak PAI-1 expression was an independent marker for lymph node metastases, with approximately 8-fold increased risk when compared to strong expression (OR = 8.60, CI = 1.54–48.08, p = 0.014, Table 3). Besides that, the lymphatic invasion also was correlated with the lymph node status (OR = 17.87, CI = 1.83–174.19, p = 0.013, Table 3).

Disease-Free Survival, Local Disease-Free Survival, and Disease-Specific Survival curves were obtained but p-values were not significant (data are not shown).

4. Discussion

In this research, PAI-1 expression in mononuclear inflammatory cell
infiltrate is a potential marker for lymph node metastases in OSCC, in which weak positive expression is associated with an approximately 8-fold increased risk. The association of PAI-1 and VEGFA also demonstrated the predictive potential of the marker in metastatic conditions.

The importance of PAI-1 in tumorigenesis is related to the role in the metastatic cascade, regulating the proliferation, migration, invasion, and adhesion of tumors cells by inhibition of uPA/uPAR complex [24, 25], in which PAI-1 participates in the clinical outcome unsatisfactory in patients with breast, ovarian, bladder, colon cancer [26].

The action of PAI-1 on inflammation is related to the activation of the hemostatic system that directs the restructuring of tissue functions after injury. In this, inflammatory mediators such as interleukin 1 (IL-1), interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α) and inflammatory cytokines act on activation of fundamental mechanisms that involve antifibrinolytics release, including PAI-1, in the tissue undergoing recovery [25-30]. In this sense, low levels of PAI-1 may indicate a failure in the tissue homeostasis recovery system and may be indicative of worse prognosis.

Barajas-Castañeda et al. [31] reported that in metastatic tumors have a decrease of PAI-1 expression, suggesting a specific regulatory system for the activation of the metastatic chain, with the involvement of the fibrinolytic pathway mediated by uPA, uPAR, and PAI-1. Another study shows the involvement of inflammatory response, neoplastic cells, and lymph nodes in the evolution of metastatic processes, these together which may indicate disease progression, worse prognosis, and decrease survival in patients diagnosed with head and neck cancer [32].

Among inflammatory responses, platelet release reaction has a relevant role and can be activated by proinflammatory and procoagulant substances, such as growth factors and PAI-1, respectively [33]. In this perspective, the positive correlation between strong PAI-1 expression in the intratumoral inflammatory infiltrate and vascular endothelial growth factor (VEGFA), shows that PAI-1 is a potential marker for a tissue homeostasis recovery system, and its expression indicates the bidirectional balancing between inflammation and homeostasis.

The finding also corroborates to the discussions about heterotypic interactions that occur in the process of tumorigenesis [34], in which the cancer cells when do not find the necessary resources for survival and development, related to the high levels of PAI-1 in the inflammatory infiltrate, starting the process of intratumoral heterogeneity and secretion of angiogenic factors to improve the nutrient and oxygen supply, and guarantee proliferation and survival [35].

The multivariate analysis also showed a positive association between the presence of lymphatic invasion and affected lymph nodes, and this can be justified by the composition of tumor microenvironment in response to inflammation, in which it is directly related to the production of angiogenic factors, proteases and growth factors, resulting in an environment that stimulates migration, proliferation and epithelial cells survival [36,37]. This hypothesis also explains the strong expression of PAI-1 in the inflammatory infiltrate found in this study: the lymphoid cells show of increase of the expression of PAI-1 as a response to start the metastatic cascade in oral squamous cell carcinoma.

Although no correlation was observed between PAI-1 in monocellular inflammatory cell infiltrate and PAI-1 cytoplasmic and membrane tumor expressions, previous research of our group indicated the relation between tumor PAI-1 expression and invasion processes, in association with others markers of hypoxia, such as VEGFA [21]. Furthermore, in this previous research, PAI-1 proved to be a promising marker for understanding the expression of tissue factors and procoagulant induced by inflammatory stimuli.

This study strengthens research that links inflammation with worse clinical outcomes among patients, as well as corroborates the reported correlation between the expression of PAI-1 for other types of neoplasms such as breast, ovary, bladder, colon cancer and non-small cell
5. Conclusion

This study was able to analyze and categorize the expression of the PAI-1 in mononuclear inflammatory cell infiltrate in OSCC, in which the high levels of PAI-1 act in the reduction of fibrinolytic activity and of metastatic evolution. Therefore, this strong PAI-1 expression is an indicator of a better prognosis in OSCC.

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Provenance and peer review

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Declaration of competing interest

The authors declare no conflict of interest.

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Table 2
Clinical and pathological features and their relation with PAI-1 expression in the mononuclear cell of the intratumoral inflammatory infiltrate.

| Clinical and pathological features | Total | Weak | Strong | p-value |
|-----------------------------------|-------|------|--------|---------|
|                                   | No. (%)| No. (%)| No. (%)|         |
| Tumor size (T)
| T1 + T2 19 (48.7) 8 (57.1) 11 (44.0) 0.475 |
| T3 4 (10.3) 2 (14.3) 2 (8.0) |
| T4 16 (41.0) 4 (28.6) 12 (48.0) |
| Lymph node (N)
| Negative 22 (56.4) 4 (28.6) 18 (72.0) 0.009 |
| Positive 17 (43.6) 10 (71.4) 7 (28.0) |
| Differentiation
| Well 18 (46.2) 8 (57.1) 10 (40.0) 0.303 |
| Moderately 21 (53.8) 6 (42.9) 15 (60.0) |
| Lymphatic invasion
| Absent 29 (74.4) 10 (71.4) 19 (76.0) 0.754 |
| Present 10 (25.6) 4 (28.6) 6 (24.0) |
| Perineural invasion
| Absent 21 (53.8) 10 (71.4) 11 (44.0) 0.099 |
| Present 18 (46.2) 4 (28.6) 14 (56.0) |
| PAI-1 cytoplasmic tumor expression
| Negative 7 (17.9) 5 (35.7) 2 (8.0) 0.075 |
| Positive 32 (82.1) 9 (64.3) 23 (92.0) |
| VEGFA cytoplasmic tumor expression
| Negative 17 (43.6) 9 (64.3) 8 (32.0) 0.028 |
| Positive 21 (53.8) 4 (28.6) 17 (68.0) |
| Not available 1 (2.6) 1 (7.1) 0 (0.0) |
| Disease relapse
| No 22 (56.4) 9 (64.3) 13 (52.0) 0.542 |
| Present 16 (41.0) 5 (35.7) 11 (44.0) |
| Not available 1 (2.6) 0 (0.0) 1 (4.0) |
| Disease-specific death
| No 21 (53.8) 7 (50.0) 14 (56.0) 0.342 |
| Yes 14 (35.9) 7 (50.0) 7 (28.0) |
| Not available 4 (10.3) 0 (0.0) 4 (16.0) |
| Total 39 (100.0) 14 (35.9) 25 (64.1) |

Table 3
Multivariate analysis of the relationship between lymph nodes and PAI-1 expression in the mononuclear cell of the intratumoral inflammatory infiltrate, tumor size, and lymphatic invasion.

| Variables | Multivariate analysis |
|-----------|----------------------|
| OR (95% CI) | p-value |
| Lymphnode (N)
| Negative 8.60 (1.54–48.08) 0.014 |
| Positive 1 |
| Tumor size (T)
| T1 0.97 (0.09–10.24) 0.982 |
| T2 1.06 (0.04–7.71) 0.974 |
| T4 0.54 (0.04–7.10) 0.642 |
| Lymphatic invasion
| Absent 17.87 (1.83–174.19) 0.013 |
| Present 1 |

Table 4
Clinical and pathological features and their relation with PAI-1 expression in the mononuclear cell of the intratumoral inflammatory infiltrate.

| Clinical and pathological features | Total | Weak | Strong | p-value |
|-----------------------------------|-------|------|--------|---------|
|                                   | No. (%)| No. (%)| No. (%)|         |
| Tumor size (T)
| T1 + T2 19 (48.7) 8 (57.1) 11 (44.0) 0.475 |
| T3 4 (10.3) 2 (14.3) 2 (8.0) |
| T4 16 (41.0) 4 (28.6) 12 (48.0) |
| Lymph node (N)
| Negative 22 (56.4) 4 (28.6) 18 (72.0) 0.009 |
| Positive 17 (43.6) 10 (71.4) 7 (28.0) |
| Differentiation
| Well 18 (46.2) 8 (57.1) 10 (40.0) 0.303 |
| Moderately 21 (53.8) 6 (42.9) 15 (60.0) |
| Lymphatic invasion
| Absent 29 (74.4) 10 (71.4) 19 (76.0) 0.754 |
| Present 10 (25.6) 4 (28.6) 6 (24.0) |
| Perineural invasion
| Absent 21 (53.8) 10 (71.4) 11 (44.0) 0.099 |
| Present 18 (46.2) 4 (28.6) 14 (56.0) |
| PAI-1 cytoplasmic tumor expression
| Negative 7 (17.9) 5 (35.7) 2 (8.0) 0.075 |
| Positive 32 (82.1) 9 (64.3) 23 (92.0) |
| VEGFA cytoplasmic tumor expression
| Negative 17 (43.6) 9 (64.3) 8 (32.0) 0.028 |
| Positive 21 (53.8) 4 (28.6) 17 (68.0) |
| Not available 1 (2.6) 1 (7.1) 0 (0.0) |
| Disease relapse
| No 22 (56.4) 9 (64.3) 13 (52.0) 0.542 |
| Present 16 (41.0) 5 (35.7) 11 (44.0) |
| Not available 1 (2.6) 0 (0.0) 1 (4.0) |
| Disease-specific death
| No 21 (53.8) 7 (50.0) 14 (56.0) 0.342 |
| Yes 14 (35.9) 7 (50.0) 7 (28.0) |
| Not available 4 (10.3) 0 (0.0) 4 (16.0) |
| Total 39 (100.0) 14 (35.9) 25 (64.1) |

a TNM classification 8th edition [19].
b Not available (not considered in the statistical calculations).

lign [38–41].

5. Conclusion

This study was able to analyze and categorize the expression of the PAI-1 in mononuclear inflammatory cell infiltrate in OSCC, in which the...
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