

# Expression of urokinase-type plasminogen activator and its receptor in squamous cell carcinoma of the oral tongue

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**Abstract:** Urokinase-type plasminogen activator (uPA) and its receptor (uPAR) act in the proteolysis of basement membrane and extracellular matrix structures, facilitating tumor invasion. The purpose of this study was to evaluate the relationship between these proteins and clinicopathological parameters in squamous cell carcinoma of the oral tongue (SCCOT). Sixty cases of SCCOT were submitted to immunohistochemistry and analyzed semiquantitatively at the invasion front and in the tumor core. The results were associated with lymph node metastasis, clinical stage, locoregional recurrence, clinical outcome and histological grade of malignancy. A higher expression of uPA was observed in cases of tumors of high-grade versus low-grade malignancy ( $p = 0.010$ ). Moreover, the cases with the worst pattern of invasion presented an overexpression of uPA ( $p = 0.011$ ). The presence of locoregional recurrence was associated with uPAR ( $p = 0.039$ ), and the expression of both biomarkers was much higher at the invasion front than in the tumor core ( $p < 0.001$ ). The results suggest uPA and uPAR are involved in the progression and aggressiveness of SCCOT, mainly at the tumor-host interface.

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## Introduction

The plasminogen activator system (PAS) is responsible for the conversion of the pro-enzyme plasminogen into serine protease plasmin. Plasmin not only plays a role in the fibrinolysis of blood clots and the restoration of blood flow, but also acts pathophysiologically in several other processes, such as cell migration, angiogenesis, tissue remodeling and tumor cell invasion.<sup>1,2</sup>

In carcinogenesis, plasmin directly degrades components of the basement membrane and interstitial extracellular matrix, such as laminin, collagen IV and fibronectin, or indirectly degrades them by the activation of other proteinases like matrix metalloproteinases (MMP).<sup>3,4</sup> Moreover, plasmin can activate fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and transforming growth factor- $\beta$  (TGF- $\beta$ ), which all favor tumor growth and invasion.<sup>3,5</sup>

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Two types of PAS activators are responsible for the formation of plasmin: tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). tPA acts exclusively on fibrinolysis and uPA binds to its specific receptor, the urokinase-type plasminogen activator receptor (uPAR), to promote tissue repair.<sup>4,6</sup> Increased expression of uPA/uPAR has been observed in some tumors, including those in stomach,<sup>7</sup> colorectal<sup>8</sup> and oral cavity.<sup>9,10,11</sup>

Oral squamous cell carcinoma (OSCC) is a common tumor with a 5-year survival rate that ranges from 15-50%.<sup>12,13</sup> The oral tongue (SCCOT) is the most common site, representing 25-40% of the cases.<sup>14</sup> This anatomic location has an enhanced risk of recurrence and lymph node metastasis due to its rich lymphatic network and extensive muscular area.<sup>14,15</sup> The high incidence and aggressive behavior of SCCOT has warranted a special study of this lesion separately from those of other oral locations.

The objective of this study was to evaluate the immunoexpression of uPA and uPAR in SCCOT, and associate these results with clinicopathological parameters. Our aim was to gain a better understanding of the role of these proteins, and thus improve our comprehension of the biological behavior of this malignant neoplasm.

## Methodology

### Samples

All the cases of SCCOT registered at the Anatomic Pathology Service of the Dr. Luiz Antônio Hospital, Natal-RN, from 2002-2010, were evaluated. Only primary tumors treated by surgical excision without radiotherapy or previous chemotherapy were selected. Anatomopathological reports had to be made out for cases with regional lymph node metastasis. After a thorough analysis, 60 cases were included in the study.

Clinical information, such as age, gender, TNM clinical stage, presence/absence of regional lymph node metastasis, presence/absence of locoregional recurrence, and clinical outcome were obtained from the pertinent medical records. This study was approved by the Research Ethics Committee of Liga Norte-Riograndense Contra o Câncer (LNRCC) (N<sup>o</sup> 1.170.634), and complies with the Helsinki Declaration.

### Morphological analysis

All the cases were cut into 5- $\mu$ m-thick histological sections and stained by hematoxylin-eosin (HE). Two blinded trained examiners determined the histological grade of malignancy at the invasion front, based on Bryne's grading system.<sup>16</sup> The cases were classified as having a low- or high-grade of malignancy, according to the adaptation developed by Silveira et al.<sup>17</sup> In discordant cases, the evaluators reexamined the slides together to obtain a consensual result.

### Immunohistochemical reactions

The specimens were cut into 3- $\mu$ m-thick sections and placed on histological slides prepared with an organosilane-based adhesive. The sections were deparaffinized, rehydrated and immersed in 3% hydrogen peroxide. The anti-uPA (H-140, polyclonal, Santa Cruz Biotechnology, Dallas, USA) and anti-uPAR (E-3, monoclonal, Santa Cruz Biotechnology, Dallas, USA) antibodies were diluted in 1:500 and incubated at 4°C overnight. The amplification reaction was performed using the HiDef Detection HRP-Polymer System (954D; Sigma-Aldrich Co, Darmstadt, Germany) and visualized by 3,3-diaminobenzidine (Liquid DAB + Substrate, Dako North America Inc., Carpinteria, USA). Counterstaining was performed with Harris hematoxylin, and the slides were mounted in Permount® resin. Tumor tissues of OSCC that revealed high immunoexpression were the positive controls, whereas primary antibodies were replaced by 1% bovine serum albumin (BSA) in buffer solution for negative control.

### Immunohistochemical analysis

A blinded trained examiner evaluated all the cases at two distinct moments. A semiquantitative analysis of uPA and uPAR was conducted along the invasion front and the tumor core, under 40X magnification (Olympus CX31, Tokyo, Japan). An adaptation of the methodology established by Magnussen et al.<sup>11</sup> was undertaken for both biomarkers, based on the proportion of positive tumor cells, as follows: 0 = none; 1 = 1-10%; 2 = 11-50%; and 3 = > 50%. Tumor cells considered positive presented a brownish color of the membrane and/or the cytoplasm.

## Statistical analysis

The results were analyzed using the Statistical Package for the Social Sciences software (version 17.0; SPSS Inc., Chicago, USA). The non-parametric Mann-Whitney test analyzed the differences between uPA and uPAR and the clinicopathological variables. The expression of the markers in relation to the morphological parameters proposed by Bryne<sup>16</sup> was evaluated using the Kruskal-Wallis test. Statistical differences between the markers on the deep invasive front and in the tumor core were obtained using the Wilcoxon test. A significance level of 5% ( $p < 0.05$ ) was established for all the statistical tests. The kappa test evaluated the interexaminer concordance according to Bryne's grading system ( $k = 0.443$ ;  $p < 0.001$ ), as well as the intraexaminer agreement for each immunohistochemical analysis regarding the invasion front and tumor core of uPA ( $k = 0.675$  and  $0.727$ , respectively;  $p < 0.001$ ) and uPAR ( $k = 0.680$  and  $0.528$ , respectively;  $p < 0.001$ ).

## Results

### Clinicopathological results

Of the 60 cases of SCCOT included in this study, 31 (51.7%) had lymph node metastasis, and the remaining 29 (48.3%) had no such metastasis. Males predominated ( $n = 41$ ; 68.3%) and the mean age was 63.1 years. Clinical staging was categorized into two groups, with a preponderance of stages III and IV ( $n = 38$ ; 63.3%) in relation to stages I and II ( $n = 22$ ; 36.7%).

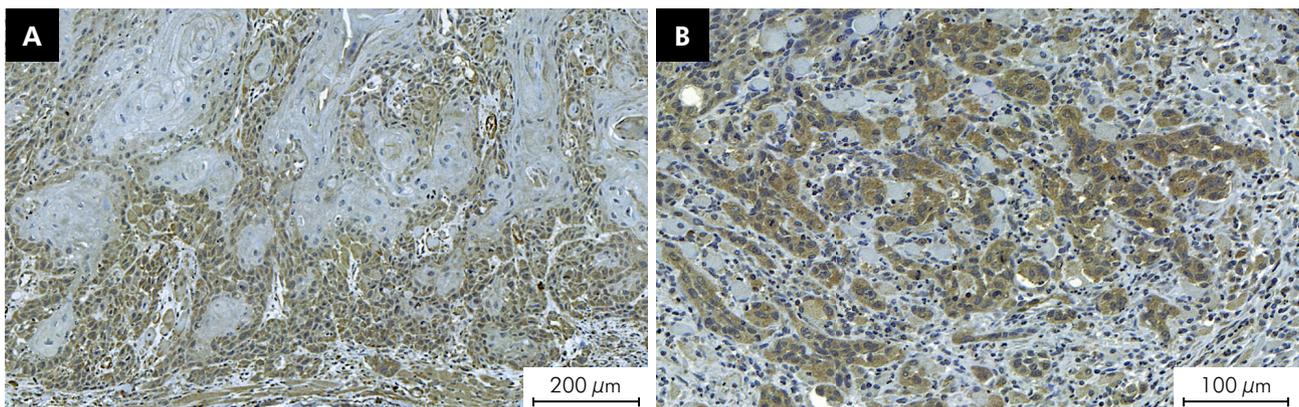
Only cases with a 5-year follow-up were considered for analysis of recurrence and outcome, yielding 46 cases. Of these, 14 (30.4%) presented recurrence. Regarding the clinical outcome, only cases of death and remission of the disease were considered. Three cases were excluded on the grounds of disease in progress. Of the 43 remaining cases, the most prevalent outcome was death ( $n = 24$ ; 55.8%) in relation to remission ( $n = 19$ ; 44.2%).

The majority of the SCCOT cases were classified as tumors of high-grade malignancy ( $n = 48$ ; 80%) in relation to low-grade malignancy ( $n = 12$ ; 20%). Most cases were highly keratinized ( $n = 25$ ; 41.7%), with moderate nuclear pleomorphism ( $n = 29$ ; 48.3%), cellular dissociation ( $n < 15$ ) ( $n = 42$ ; 70%) and moderate inflammatory infiltrate ( $n = 35$ ; 58.3%).

### Immunohistochemical results

Immunohistochemical expression of uPA was observed in the neoplastic cells of 55 cases (91.7%) at the invasion front, and in 39 cases (65%) in the tumor core (Figure 1). Most of the cases at the tumor-host interface were classified as score 1 ( $n = 23$ ; 38.3%), and only 5 cases had no staining. On the other hand, most of the cases in the tumor core were score 0 ( $n = 21$ ; 35%). The Wilcoxon test revealed a higher expression of uPA at the invasion front in relation to the tumor core ( $p < 0.001$ ) (Table 1).

The clinical parameters showed no significant differences for uPA expression (Table 2). Concerning the histological grade, most high-grade tumors had a score 2 at the deep invasive front ( $n = 17$ ; 35.4%), whereas low-grade tumors were mostly score 1 ( $n = 8$ ;



**Figure 1.** Immunohistochemical expression of uPA in SCCOT. (a) Low magnification showing the immunostaining at the invasion front and in the tumor core. (b) High magnification showing the immunostaining at the invasion front.

**Table 1.** Immunohistochemical scores of uPA according to the tumor area.

Tumor area	Immunohistochemical score [n (%)]				p
	0	1	2	3	
Invasion front	5 (8.3%)	23 (38.3%)	18 (30%)	14 (23.3%)	< 0.001*
Tumor core	21 (35%)	20 (33.3%)	10 (16.7%)	9 (15%)	

\*Statistically significant.

**Table 2.** Immunopositivity of uPA at the invasion front and in the tumor core according to clinicopathological parameters.

Location/ Parameters	Group	n	Median	Q <sub>25</sub> -Q <sub>75</sub>	p
Invasion front					
Nodal metastasis	Absent	29	1.00	1.00-2.50	0.907
	Present	31	2.00	1.00-2.00	
Clinical stage	I-II	22	1.50	1.00-2.75	0.846
	III-IV	38	2.00	1.00-3.00	
Recurrence	Absent	32	2.00	0.00-3.00	0.564
	Present	14	2.00	1.00-2.00	
Outcome	Remission	19	2.00	1.00-3.00	0.888
	Death	24	2.00	1.00-2.75	
Histological grade	Low-grade	12	1.00	1.00-2.00	0.010*
	High-grade	48	2.00	1.00-3.00	
	Bands/strands	9	1.00	1.00-1.00	0.011*
Invasion pattern	Small groups (n > 15)	9	1.00	0.50-1.50	
	Cellular dissociation (n < 15)	42	2.00	1.00-3.00	
Tumor core					
Nodal metastasis	Absent	29	1.00	0.00-2.00	0.572
	Present	31	1.00	0.00-2.00	
Clinical stage	I-II	22	1.00	0.00-2.00	0.942
	III-IV	38	1.00	1.00-2.00	
Recurrence	Absent	32	1.00	0.00-2.00	0.252
	Present	14	1.00	0.00-1.25	
Outcome	Remission	19	1.00	0.00-2.00	1.000
	Death	24	1.00	0.00-2.00	

\*Statistically significant.

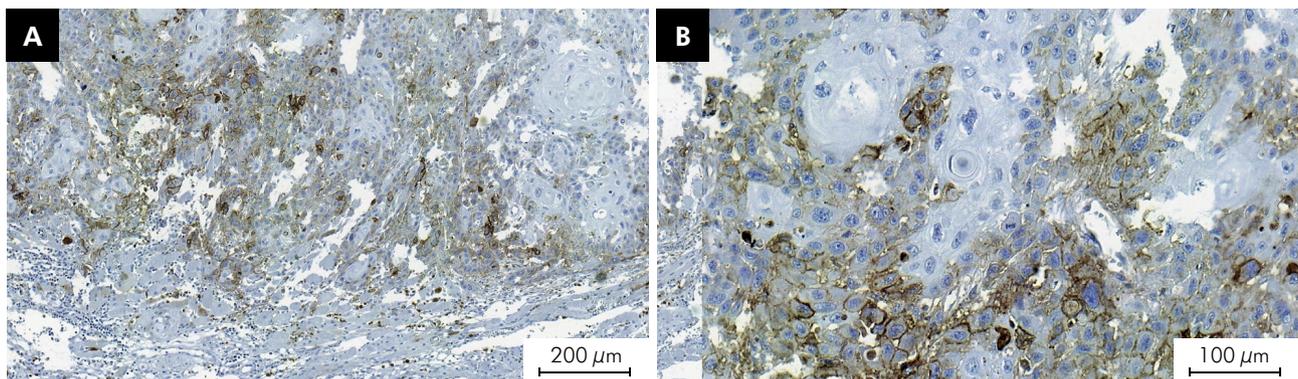
66.7%). The Mann-Whitney test revealed higher uPA expression in cases of high-grade compared to low-grade malignancy ( $p = 0.010$ ) (Table 2). When the morphological parameters were evaluated, a higher uPA expression was observed in the pattern of cellular dissociation invasion ( $n < 15$ ), in relation to the pattern of bands and/or strands and small groups ( $n > 15$ ) ( $p = 0.011$ ) (Table 2).

uPAR expression was observed in the epithelial cells of 46 cases (76.7%) at the invasion front, and in 29 cases (48.4%) in the tumor core (Figure 2). Most cases at the invasion front were classified as score 1 (46.7%), whereas most cases in the tumor core presented a score of 0 (51.7%). The Wilcoxon test revealed a significantly higher ( $p < 0.001$ ) expression of uPAR at the invasion front than in the tumor core (Table 3).

Regarding the clinical parameters, most cases at the invasion front with no locoregional recurrence presented a score of 1 ( $n = 16$ ; 50%) and 0 ( $n = 10$ ; 31.3%), whereas the cases with recurrence showed a predominance of score 1 ( $n = 5$ ; 35.7%) and 2 ( $n = 4$ ; 28.6%). The Mann-Whitney test revealed a higher uPAR expression at the invasion front in the cases with recurrence versus without recurrence ( $p = 0.039$ ) (Table 4). No statistically significant differences were observed for the other clinical parameters or the histological grade (Table 4).

## Discussion

uPA and uPAR are implicated in tumorigenesis, since they are responsible for greater infiltration



**Figure 2.** Immunohistochemical expression of uPAR in SCCOT. (a) Low magnification showing the immunostaining at the invasion front and in the tumor core. (b) High magnification showing the immunostaining at the invasion front.

**Table 3.** Immunohistochemical scores of uPAR according to the tumor area.

Tumor area	Immunohistochemical score [n (%)]				p
	0	1	2	3	
Invasion front	14 (23.3%)	28 (46.7%)	10 (16.7%)	8 (13.3%)	< 0.001*
Tumor core	31 (51.7%)	25 (41.7%)	4 (6.6%)	0 (0%)	

\*Statistically significant.

**Table 4.** Immunopositivity of uPAR at the invasion front and in the tumor core according to the clinicopathological parameters.

Location/ Parameters	Group	n	Median	Q <sub>25</sub> -Q <sub>75</sub>	p
Invasion front					
Nodal metastasis	Absent	29	1.00	1.00–2.00	0.196
	Present	31	1.00	0.00–1.00	
Clinical stage	I-II	22	1.50	1.00–2.00	0.052
	III-IV	38	1.00	1.00–1.75	
Recurrence	Absent	32	1.00	0.00–1.00	0.039*
	Present	14	1.50	1.00–2.00	
Outcome	Remission	19	1.00	1.00–3.00	0.392
	Death	24	1.00	1.00–2.75	
Histological grade	Low-grade	12	1.00	1.00–2.00	0.229
	High-grade	48	1.00	1.00–2.00	
	Bands/strands	9	0.50	0.00–1.00	0.222
Invasion pattern	Small groups (n > 15)	9	1.00	0.00–2.00	
	Cellular dissociation (n < 15)	42	1.00	1.00–2.00	
Tumor core					
Nodal metastasis	Absent	29	1.00	0.00–1.00	0.635
	Present	31	0.00	0.00–1.00	
Clinical stage	I-II	22	1.00	0.00–1.00	0.201
	III-IV	38	0.50	0.00–1.00	
Recurrence	Absent	32	0.00	0.00–1.00	0.214
	Present	14	1.00	1.00–2.25	
Outcome	Remission	19	0.00	0.00–1.00	0.179
	Death	24	1.00	0.00–1.00	

\*Statistically significant.

of malignant cells into adjacent tissues by plasmin activation.<sup>2,3</sup> In addition, uPA/uPAR interaction stimulates signaling pathways that maintain the mitogenic activity of cancer cells, such as focal adhesion kinase (FAK) and extracellular-signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK).<sup>18,19</sup> According to Lester et al.,<sup>20</sup> uPAR is also involved in epithelial-mesenchymal transition by activation of ERK, phosphoinositide 3-kinase/

protein kinase B (PI3K/Akt) and proto-oncogene tyrosine-protein kinase (Src) pathways, under hypoxic conditions.

Previous studies<sup>21,22</sup> on different tumors found that uPA is overexpressed in advanced tumors with lymph node metastasis and recurrence. Regarding OSCC, Yoshiwaza et al.<sup>9</sup> observed a higher survival rate in cases with a lower uPA expression. Zhang et al.<sup>10</sup> reported that uPA was more common in cases of SCCOT with lymph node metastasis and stages III-IV, confirming that uPA appears to play a role in neoplastic progression.

In the present study, no statistical significance was observed between uPA and clinical parameters. Similarly, another study<sup>23</sup> on OSCC revealed no association of this protein with lymph node metastasis or clinical stage. Gershtein et al.<sup>23</sup> reported that just because no association can be established does not mean that uPA is not associated with carcinogenesis. This is based on their observation of a higher uPA expression in tumor tissue in comparison with normal oral mucosal tissue, suggesting uPA is involved in OSCC. Although no association was established between uPA and clinical parameters, an evident expression of uPA was noticed in most cases of SCCOT, especially at the invasion front, suggesting its participation in this carcinoma.

Regarding histological grade, a significant expression of uPA was observed in high-grade versus low-grade tumors. Furthermore, cases with cellular dissociation (n < 15) presented an overexpression of uPA. In OSCC, similar results were reported by Yoshizawa et al.,<sup>9</sup> Nozaki et al.,<sup>24</sup> and Inoue et al.<sup>25</sup>, who showed that uPA is associated with the worst pattern of invasion, characterized by a diffuse distribution of neoplastic cords. Thus, these authors suggested uPA could serve as a biomarker to assess the progression of this neoplasm. Additionally, Nozaki et al.<sup>24</sup> reported that the association of the invasion pattern with uPA indicates the participation of this protein in the degradation of structures important for tissue maintenance.

According to Costa et al.,<sup>26</sup> the invasion pattern is a criterion of substantial prognostic value, since it reflects the cohesion between neoplastic cells. Well-differentiated OSCC invades well-defined margins, whereas anaplastic

tumors invade in small cellular aggregates or as isolated cells, characterizing a more aggressive behavior. Additionally, Manjula et al.<sup>27</sup> suggest that the invasion pattern is an important component of the histological grade of malignancy when evaluating the risk of lymph node metastasis, and may be a useful approach in treatment planning. Based on our results, we believe that higher uPA expression in cases of high-grade tumors and worst invasion pattern indicate an imperative role in the invasion of neoplastic cells in SCCOT.

In relation to uPAR, some authors observed its overexpression in OSCC, resulting in larger sizes, lymph node metastasis and advanced TNM.<sup>25,28</sup> Moreover, it was also reported to be associated with poorly differentiated cases.<sup>29</sup> Although no evidence was found in this study regarding a uPAR relationship with the clinical stage, the clinical outcome or the histological grade of malignancy, our results showed that cases with recurrence had greater immunostaining than those without recurrence, corroborating the studies by Memarzadeh et al.<sup>30</sup> and Kita et al.<sup>31</sup>, conducted in endometrial and gastric cancer, respectively. This represents an important finding, since it reveals that identification of the more aggressive cases could possibly be made by immunostaining, and shows the involvement of uPAR in the progression of this neoplasm.

A third component of the PAS has also been analyzed in some studies: the plasminogen activator inhibitor (PAI-1 or SERPINE1).<sup>11,32</sup> Initially, PAI-1 was believed to serve as a regulator, inactivating the uPA/uPAR complex when plasmin formation is overwhelmed. Nevertheless, studies have shown poor prognosis, and advanced tumors present high expression of PAI-1.<sup>33</sup> As such, this protein appears to interact with other mechanisms that are independent of uPA, such as ECM components, EMT induction and the angiogenesis process. This suggests that PAI-1 is associated with cell migration and metastasis.<sup>2,4</sup> The high presence of PAS components (uPA, uPAR and PAI-1) in tumors underscores their relevance in carcinogenesis.

Evaluation of the biomarkers in the tumor area revealed higher expression of uPA and uPAR at the invasion front in relation to the tumor core. This finding indicates that the proteins are expressed differently

depending on the intratumoral location, but both express greater activity at the tumor-host interface. To our knowledge, although other studies<sup>10,29</sup> have observed this event, ours was the first to undertake its evaluation. Our findings corroborate the concept that morphological and functional features at the deep invasion front better elucidate the aggressive behavior of OSCC. These biomarkers are part of a proteolytic system that plays a crucial role in cell invasion and in the degradation of components of the basement membrane and extracellular matrix.<sup>29</sup> As such, they are mostly present in the deepest region of the tumor. Therefore, the invasion front seems to best reflect the biological behavior of these proteins in SCCOT.

The enhanced expression of uPA/uPAR in solid tumors has made them therapeutic targets mostly focused on down-regulation or the blockade of their interaction. As an antagonist of uPA, the 8-mer capped peptide antagonist (Å6) has shown promising results inhibiting metastasis and tumor growth in models of breast cancer.<sup>34</sup> Phase I and II clinical trials in advanced gynecological cancers found Å6 to be safe and well tolerated.<sup>35,36</sup> In head and neck tumors, WX-UK1 (3-amidinophenylalanine-based inhibitor)—a serine protease inhibitor of uPA activity—diminished tumor cell invasion by 50% in cell lines.<sup>37</sup> Moreover, a Phase I trial showed that its prodrug, WX-671, was safe and mostly associated with mild and moderate adverse effects.<sup>38</sup> Combined therapies of Å6 with cisplatin,<sup>39</sup> and WX-671 with gemcitabine,<sup>40</sup> have also provided a better treatment response in glioblastoma and pancreatic cancer, respectively. The results underscore the importance of understanding uPA/uPAR mechanisms as a way to study therapeutic targets better and evaluate their clinical value adequately.

The main limitation of this study was the sample size. Since our focus was to examine tumors of only a specific location, our number of study patients was limited. In addition, other exclusion criteria, such as previous history of radiotherapy or chemotherapy, incomplete medical records and insufficient tumor tissue for histological and immunohistochemical analysis, further reduced the number of cases. On the other hand, we were able to obtain relevant information regarding SCCOT by the end of the sample selection.

## Conclusions

Both uPA and uPAR appear to regulate tumor invasion, and are involved in the progression of SCCOT toward a poor prognosis. In general, the high expression

of uPA observed in high-grade tumors and in the worst pattern of invasion, and the high expression of uPAR in cases with locoregional recurrence, suggest that these markers are involved in the aggressiveness of SCCOT, and act mainly at the tumor-host interface.

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