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Characterization of mast cell subpopulations in lip cancer

I. G. Rojas[1], M. L. Spencer[2,3], A. Martínez[2], M. A. Maurelia[1], M. I. Rudolph[4]

[1]*Department of Oral Surgery, College of Dentistry, Universidad de Concepción, Concepcion, Chile;* [2]*Department of Oral Pathology, College of Dentistry, Universidad de Concepción, Concepcion, Chile;* [3]*Department of Anatomical Pathology, College of Medicine, Universidad de Concepción, Concepcion, Chile;* [4]*Department of Pharmacology, College of Biological Sciences, Universidad de Concepción, Concepcion, Chile*

BACKGROUND: Lip squamous cell carcinoma (SCC) is the most common form of oral cancer. Human mast cells (MCs), which are increased in lip SCC, are classified by their protease content in tryptase-positive (MC_T) and tryptase/chymase-positive (MC_{TC}). MC proteases are associated with tumor progression and angiogenesis. The aim of this study was to quantify and characterize MC subpopulations in lip SCC.

METHODS: Serial sections from lip SCC ($n = 21$) and normal lip vermilion ($n = 8$) biopsies were stained immunohistochemically for tryptase and enzymehistochemically for chymase to determine MC subpopulation density and distribution.

RESULTS: MC_T and MC_{TC} were increased in lip SCC when compared with normal lip ($P < 0.0001$), where MC_T predominated over MC_{TC} ($P < 0.01$). In lip SCC neither subpopulation predominated. Regarding distribution, MC_T were higher than MC_{TC} at the intratumoral stroma, whereas MC_{TC} were higher than MC_T at the peritumoral stroma ($P < 0.01$).

CONCLUSIONS: The results suggest that MC subpopulations may contribute to lip SCC progression. While intratumoral MC_T may stimulate angiogenesis, peritumoral MC_{TC} may promote extracellular matrix degradation and tumor progression at the invasion front.

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Keywords: chymase; lip cancer; mast cells; proteases; tryptase

Introduction

Squamous cell carcinoma (SCC) of the lip is the most common form of oral cancer. It affects mainly older, light-skinned males chronically exposed, among other factors, to ultraviolet sunlight (1, 2). Countries like Chile, located near the Antarctic ozone hole, have experienced an increasing incidence of different forms of skin cancers, including lip cancer (3).

Several studies have shown that mast cells (MCs) are significantly increased in several neoplasias, including oral, skin, breast, and cervical cancer (4–7). Furthermore, MC density has been associated with bad prognosis and increased metastasis (6). In oral SCC an association between MCs and angiogenesis has been described (4). Several MC products can contribute to tumor angiogenesis and spreading, including the serine proteases tryptase and chymase (5, 8, 9). Human MCs are classified according to protease content in MC_T, if they only contain tryptase, and in MC_{TC} if they contain both tryptase and chymase (9, 10). MC_T have been associated with increased angiogenesis (11), and are found in higher numbers than MC_{TC} in uterine cancer (7). MC_{TC} are associated with extracellular matrix degradation and angiogenesis, and are found increased during wound healing (12). In addition, increased MC_{TC} have been linked with bad prognosis in lung adenocarcinoma (13). As differences in MC subpopulations have been found in several malignant lesions when compared with normal tissue (6, 7, 13), the aim of this study was to characterize the MC subpopulations present in lip SCC and normal lip in a sample of the Chilean population.

Material and methods

Lip biopsies

Biopsies of lip vermilion ($n = 21$, six women and 15 men; age: 35–84 years, mean: 64 ± 13 years) with histologically confirmed primary, well-differentiated, invasive lip SCC were obtained from the Archives of the Oral Pathology Laboratory, Facultad de Odontología, and the Archives of the Pathological Anatomy Department, Facultad de Medicina, Universidad de Concepción. Normal lip vermilion biopsies were also evaluated (two women and six men; age: 25–63 years, mean: 38 ± 14 years). Informed consent was obtained from all subjects. This study was approved by the Ethics Committee of the Universidad de Concepción. All

Correspondence: Dr Isolde Gina Rojas DDS, PhD, Departamento de Estomatología Quirúrgica, Facultad de Odontología, Universidad de Concepción, Casilla 160-C, Concepción, Chile. Tel.: +56 41 207122. Fax: +56 41 243311. E-mail: grojas@udec.cl
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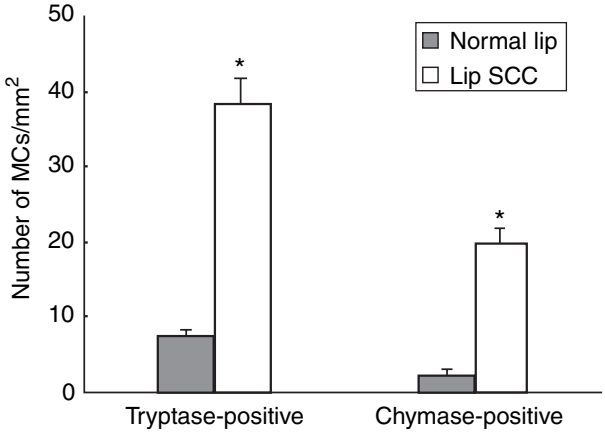

Figure 1 Mast cell density in lip squamous cell carcinoma (SCC) and normal lip. Slides were processed for immunohistochemical detection of tryptase-positive mast cells (MCs) (also used for total MCs), and for enzymehistochemical detection of chymase-positive MCs. Results are expressed as mean MCs/mm^2 ± SEM ($n = 21$ for lip SCC samples, and $n = 8$ for normal lip samples). *$P < 0.0001$ (t-test), when compared with normal lip.

specimens were fixed in 10% buffered formalin (pH 7.4) and paraffin-embedded within 24 h. Serial sections, 4 μm thick, were taken from the tissue blocks and processed for immunohistochemical studies.

Immunohistochemical staining of tryptase-positive mast cells

Tissue sections were stained for tryptase-positive MCs as previously described (14). Briefly, sections were dewaxed and rehydrated, followed by endogenous peroxidase activity blockage with 3% H_2O_2 in absolute methanol and incubation with 10% goat serum in 1% bovine serum albumin–Tris buffered saline (BSA-TBS). Then, sections were exposed to the primary antibody specific to mast cell tryptase (15), monoclonal IgG1 mouse-anti human mast cell tryptase (1:2000) (MAB 1222, Chemicon Int., Temecula, CA, USA), in 1% BSA-TBS, followed by incubation with the secondary antibody, goat anti-mouse IgG-poly-HRP (Chemicon Int.). The reaction was developed with 3-3′-diaminobenzidine (DAB) (Chemicon Int.) and 3 ml/ml H_2O_2 in 50 mM

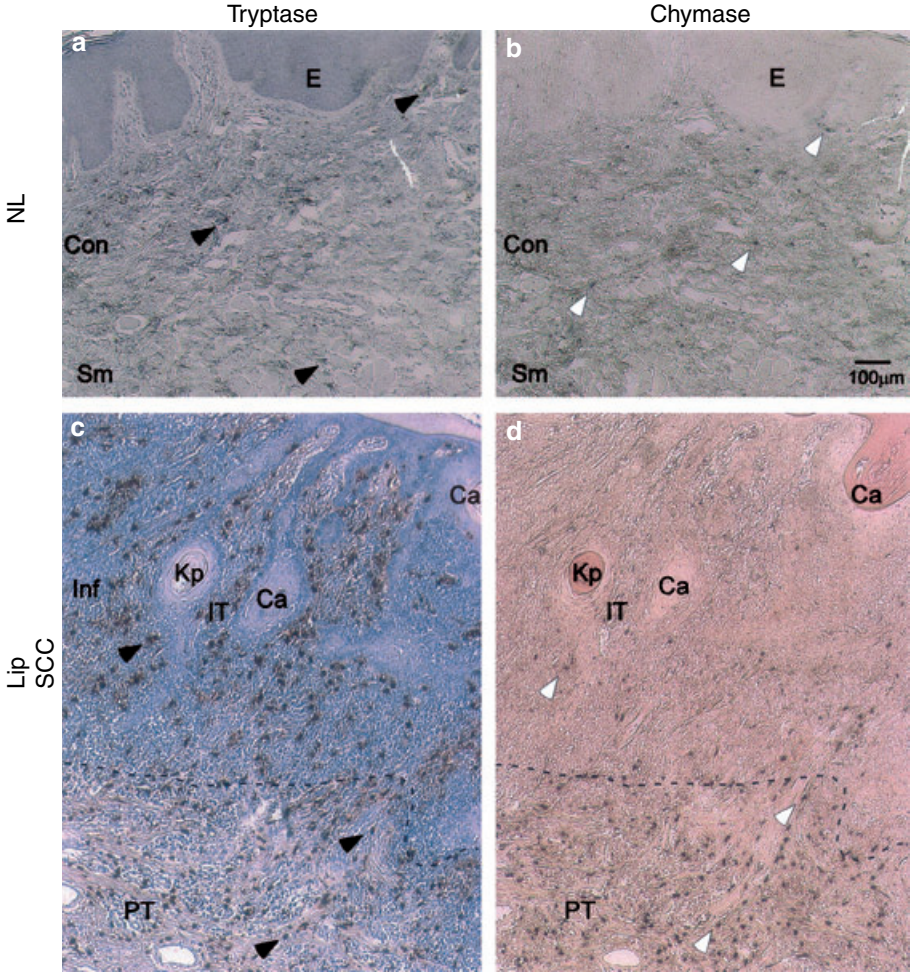

Figure 2 Staining of tryptase- and chymase-positive mast cells (MCs) in serial sections of lip squamous cell carcinoma (SCC) and normal lip. Immunohistochemical staining of tryptase-positive MCs is shown for (a) normal lip and (c) lip SCC. Enzymehistochemical staining of chymase-positive MCs is shown for (b) normal lip and (d) lip SCC. Tryptase-positive MCs are indicated with black arrowheads and chymase-positive MCs are indicated with white arrowheads. Dashed line indicates approximate limit between intratumoral (IT) and peritumoral (PT) stroma. NL, normal lip; E, epithelium; Con, connective tissue; Sm, submucous tissue; Kp, keratin pearl; Ca, cancer cells; Inf, inflammatory infiltrate.

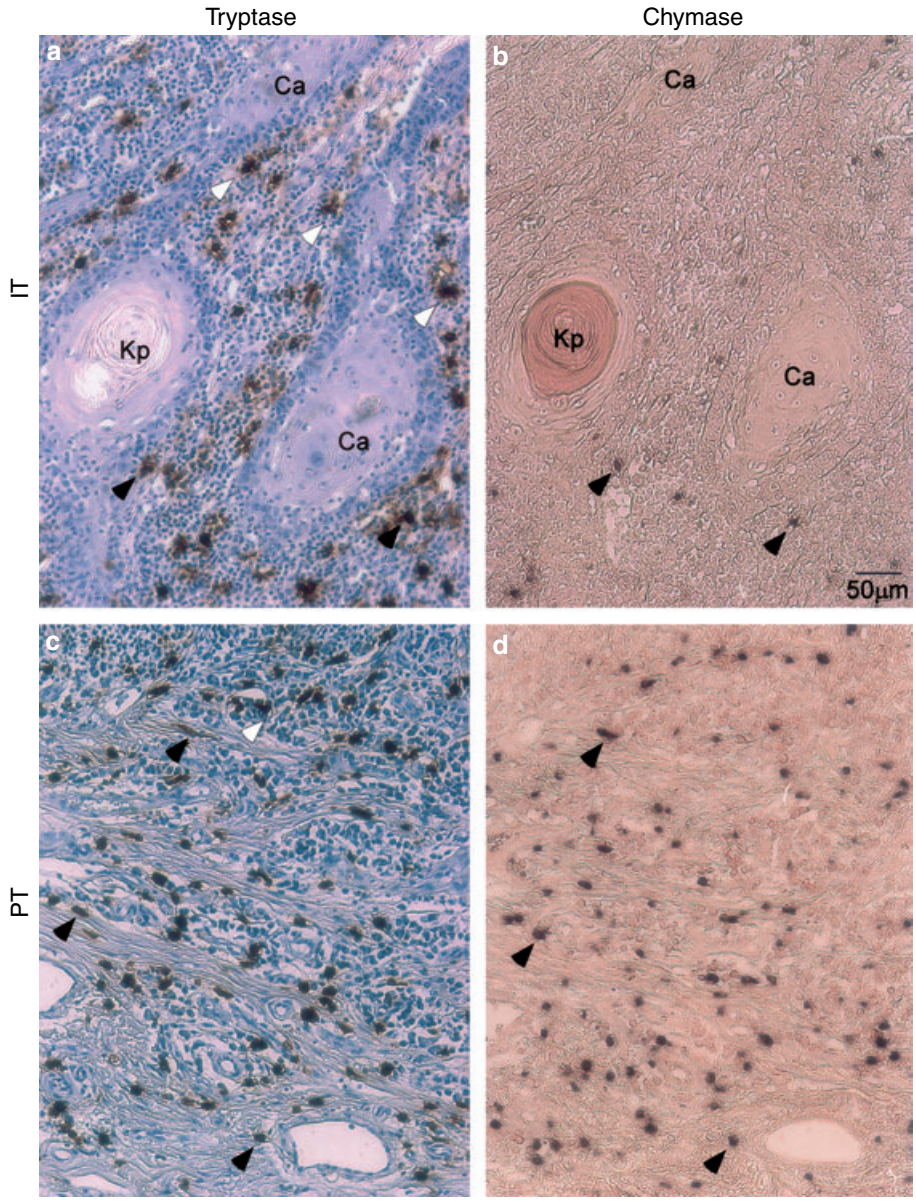

Figure 3 Distribution of tryptase- and chymase-positive mast cells (MCs) in serial sections of lip squamous cell carcinoma (SCC). Immunohistochemical staining of tryptase-positive MCs is shown for (a) intratumoral (IT) and (c) peritumoral (PT) stroma. Enzymehistochemical staining of chymase-positive MCs is shown for (b) IT and (d) PT stroma. Black arrowheads indicate MCs positive for both tryptase and chymase, and white arrowheads indicate MCs positive for tryptase only. Kp, keratin pearl; Ca, cancer cells.

Tris (pH 7.6). Slides were counterstained with Meyer's hematoxylin, dehydrated and mounted. Between steps, slides were washed three times in 1% BSA-TBS. Uterine cancer sections were used as positive control (7), and the non-specific IgG1 mouse monoclonal antibody (12CONT01, Chemicon Int.) was used as a negative control.

Enzymehistochemical staining of chymase-positive mast cells

Chymase-positive MCs were stained by using the substrate *N*-acetyl-l-methionine alpha-naphtyl ester (u–N–O–Met) as previously described (16). Briefly, sections were deparaffinized, rehydrated, and washed in 0.15 M phosphate buffer, pH 7.1. Sections were then incubated at room temperature with a solution of 5 mg of u–N–O–Met dissolved in 0.2 ml dimethyl formamide (Sigma, St Louis, MO, USA), made up to a final volume of 25 ml with 0.15 M phosphate buffer, containing 3.2 mM Fast Blue salt (Sigma) as a capture reagent. The reaction was stopped by incubation with 1% cupric sulfate. The slides were then washed with phosphate buffer, counterstained with saphranine-*O*, and mounted in Aquatex (MERCK, Darmstadt, Germany). As a negative control, the substrate was omitted from the incubation mixture.

Mast cell counting

Tryptase- and chymase-positive cells were counted separately in serial sections of normal lip and lip SCC

using a Nikon Diaphot 300 microscope (Nikon, Melville, NY, USA) equipped with an OC-M calibrated eyepiece micrometer and connected to an IMAGEPRO analysis program 4.0.1 (Media Cibernetics, Atlanta, GA, USA). MCs were counted in 30 counting fields in normal lip sections (including epithelium/connective tissue junction, connective, and submucous zones) and in 20 counting fields per lip SCC section at 40× magnification (counting field area = 0.4 mm^2). In order to assess distribution of MCs in lip SCC, 10 adjacent counting fields were located at the intratumoral stroma (where the tumor parenchyma was located) and 10 counting fields were located at the tumor invasion zone or peritumoral stroma, as previously described (6). This task was performed by two calibrated observers blinded to the objectives of the study. Results were expressed as MCs/mm^2 (mean ± SEM).

Statistical analysis

All data were tabulated and statistical tests were performed with JMP-IN 3.2.1 (SAS Institute Inc., Cary, NC, USA). Significant statistical differences between groups were examined using unpaired *t*-test. The non-parametric Wilcoxon test was used when variables did not have a normal distribution. Differences were considered statistically significant when $P < 0.05$.

Results

Determination of mast cell density and morphology in lip SCC and normal lip

MC density and protease content were determined by enzymehistochemical staining of chymase and immunohistochemical staining of tryptase. As all MCs contain tryptase (MCs containing only chymase are extremely scarce) (10, 17), tryptase-positive MCs also represented total MCs. The results showed that both tryptase- and chymase-positive cells were significantly increased in lip SCC when compared with normal lip ($P < 0.0001$, *t*-test, Figs 1 and 2).

Regarding MC location and morphology, in normal lip, MCs looked small, without signs of activation, and were found located around blood vessels (Fig. 2), as previously described (4). In lip SCC MCs looked enlarged and in a state of degranulation, especially tryptase-positive MCs (Fig. 2).

Density and distribution of mast cell subpopulations in lip SCC

To determine MC subpopulations (MC_T, MC_{TC}) in normal lip and lip SCC, the formula: Total MCs = MC_T + MC_{TC} was used as previously described (7), where Total MCs = tryptase-positive MCs and MC_{TC} = chymase-positive MCs. Results were confirmed by co-localization of serial sections stained either for tryptase- or chymase-positive MCs (Figs 2 and 3). In normal lip, the MC_T subpopulation predominated over MC_{TC} ($P < 0.02$, Wilcoxon test) as previously described (14) (Fig. 4). On the other hand, both MC subpopulations were significantly increased in lip SCC lesions when compared with normal lip ($P < 0.0001$, Wilcoxon test), but neither MC_T nor MC_{TC} predominated (Fig. 4).

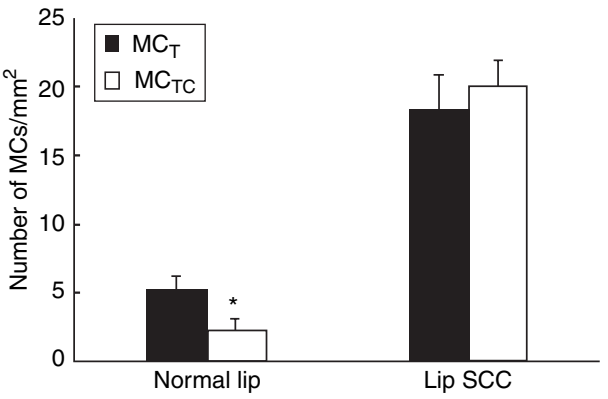

Figure 4 Density of mast cell (MC) subpopulations in lip squamous cell carcinoma (SCC). MC subpopulations were determined using the formula Total MCs = MC_T + MC_{TC}. Results are expressed as mean MCs/mm^2 ± SEM ($n = 21$ for lip SCC samples, and $n = 8$ for normal lip samples). *$P < 0.02$ (Wilcoxon) when compared with MC_T in normal lip.

To study distribution of total MCs and their subpopulations in lip SCC, MC density was determined in two specific areas, the intratumoral stroma (where the tumor parenchyma was located) and the peritumoral stroma or invasion zone (Figs 2 and 3). It was found that total MCs and MC_{TC} were significantly increased at the peritumoral stroma when compared with the intratumoral zone, while MC_T were evenly distributed in both areas (Fig. 5).

When MC subpopulations were compared within the intratumoral and peritumoral areas of the lip SCC lesions, it was found that MC_T predominated over MC_{TC} at the intratumoral stroma ($P < 0.02$, Wilcoxon test), whereas, MC_{TC} predominated over MC_T at the peritumoral zone ($P < 0.01$, *t*-test) (Figs 3 and 6).

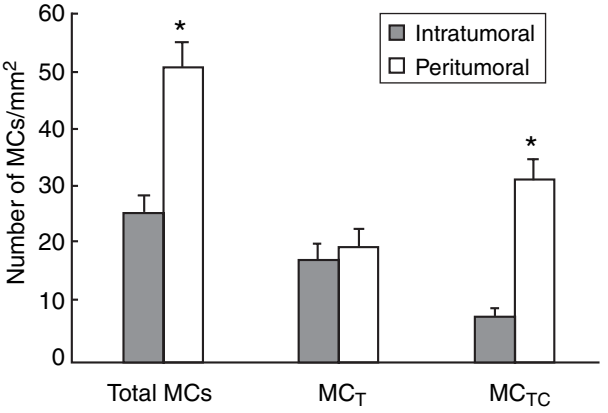

Figure 5 Distribution of mast cell (MC) subpopulations in lip squamous cell carcinoma (SCC). Number of total MCs, MC_T, and MC_{TC} were determined at the intratumoral and peritumoral stroma. Results are expressed as mean MCs/mm^2 ± SEM ($n = 21$ for lip SCC samples, and $n = 8$ for normal lip samples). *$P < 0.0001$ (Wilcoxon) when compared with the intratumoral stroma.

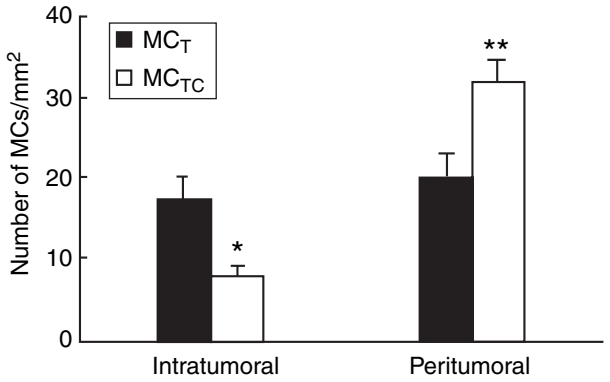

Figure 6 Predominant mast cell (MC) subpopulations within the intratumoral and peritumoral stroma. MC_T and MC_{TC} were compared at the intratumoral and peritumoral stroma of lip squamous cell carcinoma (SCC) samples. Results are expressed as mean MCs/mm^2 ± SEM ($n = 21$ for lip SCC samples, and $n = 8$ for normal lip samples). *$P < 0.02$ (Wilcoxon) when compared with MC_T at the intratumoral stroma. **$P < 0.01$ (t-test) when compared with MC_T at the peritumoral stroma.

Discussion

As previously described (4, 18), MCs were found activated and significantly increased in lip SCC lesions when compared with normal lip (eightfold increase). Significant changes in MC subpopulations in lip SCC when compared with normal lip were also found. MC_T were the predominant phenotype in normal lip, whereas in lip SCC, both MC_T and MC_{TC} were significantly increased, and MC_T did not predominate over MC_{TC}. Regarding MC distribution in lip SCC, MCs were significantly increased at the peritumoral stroma or invasion zone when compared with the intratumoral stroma. However, MC_T predominated over MC_{TC} at the intratumoral stroma, whereas MC_{TC} predominated over MC_T at the peritumoral area.

Previous studies by this group have shown that similarly to normal lip, MC_T predominate in the premalignant lip lesion, actinic cheilitis (14). In invasive lip SCC there is a clear change in the MC phenotype with an increase in MC_{TC}, and therefore of the mast cell protease chymase, which may be an indicator of malignancy. In addition, MC_{TC} increase at the peritumoral edge may be of significant importance for tumor invasion, as a similar MC_{TC} increase has been reported for gastrointestinal cancers and the most aggressive forms of lung adenocarcinoma (13, 19).

MC_{TC} contain chymase which is known for its ability to promote extracellular matrix (ECM) degradation and for indirectly stimulate angiogenesis (5). These responses are essential for tumor invasion and metastasis (5, 20). Chymase activates latent MMPs, including gelatinase B and pro-collagenases, which degrade components of epithelial basement membranes and ECM, respectively (21, 22). MC_{TC} also contain tryptase, which is a potent pro-angiogenic factor that also contributes indirectly to ECM degradation (23). Other potent ECM-degrading enzymes of MC_{TC} involve cathepsin G, carboxypeptidase, and the most recently discovered gelatinases A and B, which are important mediators of tumor progression and metastasis (9, 24). On the other hand, the MC_T subpopulation predominated at the intratumoral stroma, where angiogenesis is crucial for tumor cell growth and survival (25).

Therefore, the distribution of the different MC subpopulations found in lip SCC was in agreement with the functions previously described for each specific MC protease (5, 9). MC_{TC} containing chymase and tryptase were significantly increased at the tumor invasion zone where both ECM degradation and angiogenesis are required. On the other hand, MC_T with active tryptase were found at the intratumoral stroma, where angiogenesis is required. In addition, changes in the proportion of MC_T to MC_{TC} could be a useful indicator of malignancy in lip biopsies. Future studies should focus on the signals involved in the increased MC_T and MC_{TC} recruitment in lip SCC, as well as their significance for lip SCC pathogenesis.

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