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Title: Keratocystic odontogenic tumor overexpresses invadopodia-related proteins suggesting invadopodia formation.

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Corresponding Author: Dr. Andre Luis Ribeiro Ribeiro, DDS, MSc

Corresponding Author's Institution: School of Dentistry, University Center of Pará - CESUPA

First Author: Andre Luis Ribeiro Ribeiro, DDS, MSc

Order of Authors: Andre Luis Ribeiro Ribeiro, DDS, MSc; Natacha Malu M Costa, DDS, MSc student; Adriane S Siqueira, DDS, MSc; Walessa B Silva, DDS, MSc student; Maria Sueli S Kataoka, DDS, MSc, PhD; Ruy G Jaeger, DDS, MSc, PhD; Sérgio M Alves-Junior, DDS, MSc, PhD; Andrew M Smith, BSc, PhD; João de Jesus V Pinheiro, DDS, PhD

Abstract: OBJECTIVE: Keratocystic odontogenic tumor (KOT) is an odontogenic neoplasm that shows aggressive clinical behavior and local invasiveness. Invadopodia are actin-rich cellular protrusions exhibiting proteolytic pericellular activity, thereby inducing focal invasion in neoplastic cells and increasing neoplasms aggressiveness. Thus, this study aimed to evaluate immunoexpression of invadopodia-related proteins, cortactin, MT1-MMP, Tks4, and Tks5 in KOT.

STUDY DESIGN: Immunohistochemistry of 16 cases of KOT, 8 cases of calcifying cystic odontogenic tumor (CCOT) and 8 samples of oral mucosa (OM) was carried out to assess the expression of the above described invadopodia-related proteins in the basal and suprabasal layer.

RESULTS: KOT samples showed higher and significant immunoexpression of cortactin, MT1-MMP, TKs4 and TKs5 compared with CCOT and OM samples. Significant expression of all these proteins was observed in the basal layer when compared to the suprabasal layer in KOT.

CONCLUSIONS: Overexpression of cortactin, MT1-MMP, TKs4 and TKs5 was observed in KOT when compared to samples of CCOT and OM. These proteins were also overexpressed in the basal over the suprabasal layer of KOT samples. Taken together, these results suggest the participation of invadopodia-related proteins on the pathogenesis of this lesion.

### **Statement of Clinical Relevance**

Invadopodia are membrane protrusions present in tumor cells associated to aggressive behavior. Invadopodia-related proteins, cortactin, MT1-MMP, Tks4 and Tks5 were overexpressed in KOT. These results suggest invadopodia activity in KOT clinical behavior, increasing its aggressiveness.

**TITLE PAGE**

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**Authors' names:**

**André Luis Ribeiro Ribeiro<sup>a,b</sup> (double surname), DDS, MSc (Corresponding Author)**

**Natacha Malu Miranda da Costa<sup>c</sup>, DDS**

**Adriane Sousa de Siqueira<sup>d</sup>, DDS, MSc**

**Walessa Brasil da Silva<sup>c</sup>, DDS**

**Maria Sueli da Silva Kataoka<sup>c</sup>, DDS, MSc, PhD**

**Ruy Gastaldoni Jaeger<sup>e</sup>, DDS, MSc, PhD**

**Sérgio de Melo Alves-Junior<sup>c</sup>, DDS, MSc, PhD**

**Andrew M. Smith<sup>b</sup>, BSc, PhD**

**João de Jesus Viana Pinheiro<sup>c</sup>, DDS, PhD**

**Author' institutions:**

<sup>a</sup>, Department of Oral and Maxillofacial Surgery, School of Dentistry, University Center of Pará - CESUPA, Belém, Brazil.

<sup>b</sup>, Department of Microbial Diseases, Eastman Dental Institute, University College London, London, UK

<sup>c</sup>, Cell Culture Laboratory, Faculty of Dentistry, Federal University of Pará. Rua Augusto Corrêa, 01, Guamá, 66075110, Belém, PA, Brazil.

<sup>d</sup>, School of Dentistry, Positivo University. Rua Professor Pedro Viriato Parigot de Souza, 5300, Cidade Industrial, 81280-330, Curitiba, PR, Brazil.

<sup>e</sup>, Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

**Corresponding Author:**

1  
2 André Luis Ribeiro Ribeiro

3 Address: Travessa 9 de Janeiro, 827, Faculdade de Odontologia, Centro Universitário  
4 do Pará-CESUPA. Belém-PA, Brazil

5  
6 Postal code: 66013 090

7  
8 Phone number: 44 7823612356

9  
10 Email: [ribeiroalr@ig.com.br](mailto:ribeiroalr@ig.com.br)  
11  
12

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**Title:** Keratocystic odontogenic tumor overexpresses invadopodia-related proteins suggesting invadopodia formation.

## ABSTRACT

**OBJECTIVE:** Keratocystic odontogenic tumor (KOT) is an odontogenic neoplasm that shows aggressive clinical behavior and local invasiveness. Invadopodia are actin-rich cellular protrusions exhibiting proteolytic pericellular activity, thereby inducing focal invasion in neoplastic cells and increasing neoplasms aggressiveness. Thus, this study aimed to evaluate immunoexpression of invadopodia-related proteins, cortactin, MT1-MMP, Tks4, and Tks5 in KOT.

**STUDY DESIGN:** Immunohistochemistry of 16 cases of KOT, 8 cases of calcifying cystic odontogenic tumor (CCOT) and 8 samples of oral mucosa (OM) was carried out to assess the expression of the above described invadopodia-related proteins in the basal and suprabasal layer.

**RESULTS:** KOT samples showed higher and significant immunoexpression of cortactin, MT1-MMP, TKs4 and TKs5 compared with CCOT and OM samples. Significant expression of all these proteins was observed in the basal layer when compared to the suprabasal layer in KOT.

**CONCLUSIONS:** Overexpression of cortactin, MT1-MMP, TKs4 and TKs5 was observed in KOT when compared to samples of CCOT and OM. These proteins were also overexpressed in the basal over the suprabasal layer of KOT samples. Taken together, these results suggest the participation of invadopodia-related proteins on the pathogenesis of this lesion.

**KEYWORDS:** Keratocystic odontogenic tumor; odontogenic tumors; invadopodia; cortactin; membrane type I matrix metalloproteinase (MT1-MMP); Tks4; Tks5.

## INTRODUCTION

1  
2 Keratocystic odontogenic tumor (KOT) is a benign odontogenic epithelial  
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4 neoplasm with aggressive clinical behavior.<sup>1-4</sup> The local aggressiveness of KOT has  
5  
6 been related to many characteristics, including the biological behavior of its  
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8 neoplastic epithelial cells,<sup>4</sup> and genetic and epigenetic alterations<sup>3,5</sup>. For instance,  
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10 studies have found evidence of allelic loss mainly in p16, p53, PTCH, MCC, TSLC1,  
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12 LTAS2 and FHIT genes in KOT lesions.<sup>3,5</sup> Some studies have attempted to identify  
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14 the role of different molecules that can influence the clinical behavior of odontogenic  
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16 tumors,<sup>4,6,7</sup> however a full understanding regarding the mechanism related to KOT  
17  
18 pathological behavior remains unknown.  
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24 KOT is characterized by a well-defined basal layer of columnar or cuboidal  
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26 cells, with intense nuclear basophilia with polarity inversion. A thin capsule of  
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28 connective tissue with few inflammatory cells and layers of parakeratin can also be  
29  
30 found.<sup>2,4,8,9</sup> The basal layer sometimes shows a bud-shaped proliferation that may lead  
31  
32 to the formation of daughter lesions.<sup>4,8-12</sup> KOT recurrence is a clinical challenge and  
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34 occurs in nearly 23% of cases.<sup>2</sup> This high recurrence rate may be due to an increased  
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36 proliferative activity observed in KOT, revealed by overexpression of proliferation  
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38 markers such as Ki67 and proliferating cell nuclear antigen (PCNA) in KOT  
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40 compared to other odontogenic cysts and tumors.<sup>3,8</sup>  
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46 A previous study has demonstrated the overexpression of matrix  
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48 metalloproteinase-9 (MMP-9), epidermal growth factor (EGF) and tumor growth  
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50 factor-alpha (TGF- $\alpha$ ) in KOT cells.<sup>4</sup> Thus, it was inferred that elevated MMP-9, EGF  
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52 and TGF- $\alpha$  levels could promote ECM degradation and release of more growth  
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54 factors, contributing to KOT aggressiveness.<sup>4</sup> However, the mechanism of focal  
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56 invasion of this tumor remains unknown.  
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Neoplastic cells invasion involves the dissemination of tumor cells from a primary site to the surrounding tissues.<sup>13</sup> Among the mechanisms that mediate this process, invadopodia formation and its proteolytic activity over ECM molecules are of great importance.<sup>14,15</sup> Invadopodia are actin-rich membrane protrusions that arise from the ventral surface of neoplastic cells and are vertically extended towards ECM.<sup>16-18</sup> It is known that different proteins are related to invadopodia formation and activity.<sup>19</sup> Among those molecules, cortactin and membrane-type 1 matrix metalloproteinase (MT1-MMP) are recognized as pivotal players in invadopodia formation, which are induced by Tks5 and Tks4 respectively.<sup>14,19-23</sup>

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Cortactin is responsible for the regulation of actin polymerization and is involved in the initial steps of invadopodia formation.<sup>24,25</sup> MT1-MMP is a transmembrane metalloprotease that plays a major role in the extracellular matrix remodeling and is strongly associated with neoplastic growth.<sup>18</sup> These invadopodia-related structural modifications of the cytoskeleton are mediated by the interaction of membrane receptors with the extracellular matrix, a process that may activate Src family proteins, such as Tks4 and Tks5. Tks molecules are adaptor proteins that are normally distributed throughout the cytoplasm but tend to be localized in the plasma membrane in pathological conditions.<sup>25</sup> Tks5 has an important role in invadopodia elongation and is associated to cortactin.<sup>26,27</sup> Tks4, in turn, is responsible for regulating MT1-MMP localization, secretion and stabilization,<sup>21,25</sup> promoting localized ECM proteolysis and mediating tumor invasion.<sup>14</sup>

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Tks family members are found in many tissues and play several roles in different physiological processes. However, Tks4 and Tks5 are markedly overexpressed in invasive cancers cells and are associated with higher aggressiveness.<sup>20,27,28</sup> High levels of cortactin and MT1-MMP have already been

1 reported in ameloblastoma, an aggressive odontogenic neoplasm with local  
2 invasiveness and high recurrence rates.<sup>18</sup> This finding prompted us to assess whether  
3 invadopodia markers would be expressed in KOT. The presence of invadopodia  
4 proteins in KOT could provide important information regarding its pathological  
5 behaviour. Two control groups were used in this study, the calcifying cystic  
6 odontogenic (CCOT), a non-aggressive odontogenic neoplasm selected due to its  
7 indolent behavior and low recurrence rates<sup>4,6,18</sup> and samples of the health oral mucosa.  
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## 20 MATERIALS AND METHODS

### 21 *SAMPLE SELECTION AND CLASSIFICATION*

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26 Sixteen cases of KOT were retrieved from the files of the Department of Oral  
27 Pathology at the School of Dentistry, University Center of Pará - CESUPA (Belem,  
28 PA, Brazil). Eight cases of each control group were included in this study, which  
29 included samples of normal oral mucosa and the cystic variant of CCOT, the last  
30 chosen due to its similar odontogenic ectomesenchyme origin and distinct and  
31 indolent behavior, characterized by limited growth and low recurrences rates<sup>4,6,18</sup>. All  
32 samples were evaluated by two oral pathologists and only those that showed a well  
33 preserved basement membrane length of  $\geq 8$  millimetres (mm)<sup>29,30</sup> and fulfilled the  
34 histological profile of KOT and CCOT<sup>2</sup> were included in this study. Non-  
35 representative cases of both lesions and those that didn't reach the required basement  
36 membrane length were excluded. This study was approved by the Ethics Committee  
37 of the Institute of Health Sciences at Federal University of Pará (0084.0.073.000-10).  
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## **IMMUNOHISTOCHEMISTRY**

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3 Formalin-fixed, paraffin-embedded tissues were studied by  
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5 immunohistochemistry. Three-micron sections were obtained and mounted on poly-  
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7 D-lysine-coated slides (Sigma Chemical Corp., St Louis MO, USA). Sections were  
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9 dewaxed in xylene and rehydrated in graded ethanol. Antigen retrieval was carried out  
10  
11 in Pascal chambers (Dako, Carpinteria, CA, USA) for 30 seconds. Sections were  
12  
13 immersed in 3% H<sub>2</sub>O<sub>2</sub> in methanol for 20 minutes for the inhibition of endogenous  
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15 peroxidase activity and then blocked with 1% bovine serum albumin (BSA, Sigma®)  
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17 in phosphate-buffered saline (PBS) for 1 hour. The slides were incubated with  
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19 primary antibodies anti-cortactin (1:200, rabbit, Santa Cruz Biotechnology, CA,  
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21 USA), anti-Tks5 (1:100, goat, Abcam, Cambridge, MA, USA), anti-Tks4 (1:100,  
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23 rabbit, Abcam) and anti-MT1-MMP (1:50, mouse, R&D systems, Minneapolis, MN,  
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25 USA). All primary antibodies were diluted in antibody diluent solution (Dako®) and  
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27 incubated for 1 hour at room temperature. Subsequently, sections were incubated for  
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29 30 minutes with the biotin-free horseradish peroxidase (HRP)-labeled polymer of the  
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31 LSAB Detection System (Dako®). Diaminobenzidine (Sigma®) was used as a  
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33 chromogen, and the sections were counterstained with Mayer's hematoxylin  
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35 (Sigma®). Replacement of specific primary antibodies with non-immune sera served  
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37 as negative control and samples of oral squamous cell carcinoma were used as  
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39 positive control (data not shown).  
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## **INFLAMMATORY AND IMMUNOSTAINING EVALUATION**

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52 Brightfield images from ten randomly selected images from each sample were  
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54 acquired using an Axioskop 40 microscope (Carl Zeiss, Germany) equipped with a  
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56 CCD color camera (Axiocam MRc, Carl Zeiss). All images were acquired at the  
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58 same magnification (400x). The basement membrane was measured using the  
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1 freehand line tool of the software ImageJ (public domain software developed by  
2 Wayne Rasband - NIMH, NIH, Bethesda, MD, USA, <http://rsbweb.nih.gov/ij/>). The  
3 inflammation score was assessed by counting the total number of inflammatory cells  
4 adjacent to the epithelium in all 10 images. Inflammation assessment was carried out  
5 in grades, being Grade 0 - no inflammation, Grade 1 - <15 cells/field, Grade 2 - 15-50  
6 cells/field, and Grade 3 - >50 cells/field. The inflammatory score was calculated as  
7 the average of all fields examined. Samples of KOT, CCOT and OM were divided  
8 into two groups according to the inflammatory score: group A - grades 0-2 (mild-to-  
9 moderate) and group B - grade 3 (intense).<sup>31</sup> Comparison of the inflammation grades  
10 between group A and B and the immunoexpression of cortactin, MT1-MMP, Tks4,  
11 and Tks5, in basal and suprabasal layers were conducted for each tissue.

12 Immunostaining analysis was carried out for cortactin, MT1-MMP, Tks4, and  
13 Tks5. Areas of diaminobenzidine staining were separated and segmented using the  
14 color deconvolution plug-in of ImageJ. After image deconvolution, the epithelial area  
15 was assessed in two different segments that corresponded to the basal and suprabasal  
16 layers. Results were expressed as the average percentage of stained area (%) of the  
17 epithelial neoplastic tissue after adjusting for the correspondent basement membrane  
18 length. Differences in the percentages of stained areas for the basal and suprabasal  
19 epithelial layers of KOT, CCOT and OM were quantified. Image acquisition and  
20 measurement of diaminobenzidine staining were blinded before quantification by the  
21 examiner.

## 22 *STATISTICAL ANALYSIS*

23 Data were analyzed using the Graph Pad Prism 6 software (Graph Pad  
24 Software, Inc., San Diego, CA, USA). Differences between the three groups of  
25 samples were assessed using one-way ANOVA followed by the Tukey's multiple

1 comparison test. The parametric unpaired two-tailed t-test was used to analyze the  
2 differences between the basal and suprabasal layers. All tests were set to 95%  
3 confidence interval.  
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## 6 7 **RESULTS**

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10 The length of the basement membrane varied from 8.01 mm to 23.45 mm,  
11 with the mean length of 9.22 mm in KOT, 9.76 mm in CCOT and 13.54 mm in OM.  
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13 No cases presented inflammation grade 0, with inflammatory cells found in all  
14 samples. The summary of these results can be seen in Table I.  
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### 20 21 *KOT and CCOT immunoexpress cortactin, MT1-MMP, Tks4 and Tks5*

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24 Cortactin, MT1-MMP, Tks4, and Tks5 were expressed in all KOT and CCOT  
25 samples. The oral mucosa showed low expression of cortactin, MT1-MMP and very  
26 low expression of Tks4 and Tks5. KOT neoplastic cells showed cytoplasmic  
27 expression of cortactin as dots in the epithelial basal and suprabasal layers. (Fig 1A).  
28  
29 CCOT also expressed cortactin with a similar immunolocalization (Fig 1B). Small  
30 blood vessels and inflammatory cells were also positively stained for cortactin.  
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32 Expression of this protein was not constant in the OM and usually found in the basal  
33 layer of samples (Fig 1C). Comparative analyze showed differences between the three  
34 groups, with the highest expression found in KOT, followed by CCOT and OM (Fig.  
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49 Cytoplasmic staining was also observed for MT1-MMP in KOT and CCOT  
50 neoplastic cells (Fig 2A-B). A weak cytoplasmic staining of MT1-MMP was found in  
51 the OM, which was reduced compared to both odontogenic tumors (2C).  
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53 Inflammatory and endothelial cells were found to express MT1-MMP, specially in  
54 KOT and CCOT. MT1-MMP immunolocalization appeared to be more intense near to  
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1 the basement membrane in KOT. No difference was observed in the suprabasal layer  
2 between KOT and CCOT (2D).  
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5 Tks4 was immunostained with a granular pattern in the cytoplasm of KOT and  
6  
7 CCOT (Fig. 3A-B). Tks4 showed a more intense expression in the basal layer in KOT  
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9 and a discrete presence in the upper cells of the suprabasal layer in CCOT (Fig. 3A-  
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11 B). Stromal cells expressed Tks4, which was poorly or even non-expressed in OM  
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13 samples (Fig. 3C). Statistical analysis showed difference between KOT and the other  
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15 samples in basal and suprabasal layers, but no difference was detected between CCOT  
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17 and OM (Fig. 3D).  
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23 Similar to all the other proteins studied here, a cytoplasmic granular pattern of  
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25 staining was found for Tks5. In KOT, Tks5 immunostaining was more intense in the  
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27 basal layer and constantly expressed in all samples (Fig. 4A). The expression of Tks5  
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29 in CCOT was low, specially in the suprabasal layer (Fig. 4B). Expression of this  
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31 protein in OM was very low (Fig. 4C). Statistical deference was found between the  
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33 three tissues in both layers (Fig. 4D).  
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38 Although the CCOT samples also expressed cortactin, MT1-MMP, Tks4, and  
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40 Tks5, the overall expression of these proteins were reduced in CCOT and OM  
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42 compared to KOT.  
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46 In order to evaluate if there are differences between the expression of  
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48 invadopodia-related proteins in the basal and suprabasal layer, a comparison of the  
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50 expression of all proteins between these two layers was conducted. In comparison to  
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52 OM tissue cortactin, MT1-MMP, Tks4, and Tks5 were all overexpressed in the basal  
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54 layer of KOT samples, while no differences were observed in CCOT (Fig. 5). The  
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56 potential role of inflammation in the expression of cortactin, MT1-MMP, Tks4 and  
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1 Tks5 was assessed by comparing the basal and suprabasal layers of group A (mild-to-  
2 moderate) and group B (intense) for each tissue. The level of tissue inflammation had  
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4 no affect on the expression levels of cortactin, MT1-MMP, Tks4, and Tks5 in any  
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6 tissue. These results are summarized in Table I.  
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## 10 DISCUSSION

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12 Increased expression of cortactin and MT1-MMP has been related to  
13 aggressive behavior of various malignancies.<sup>14,15</sup> These proteins participate in  
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15 invadopodia assembly. Invadopodia are membrane protrusions that allow neoplastic  
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17 cells promote focal invasion. Membrane protrusions are driven by cortactin, while  
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19 MT1-MMP is a key molecule for pericellular focal proteolysis. Cortactin and MT1-  
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21 MMP are regulated by the Src-kinases, Tks5 and Tks4 respectively. Our results  
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23 showed overexpression of invadopodia-related proteins cortactin, MT1-MMP, Tks4,  
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25 and Tks5 in KOT compared to CCOT and OM. All proteins were only expressed in  
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27 the cytoplasm, with an increased expression in the basal layer of KOT samples, which  
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29 strongly suggest the formation of invadopodia in this lesion. Low or no expression of  
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31 invadopodia-related proteins was expected in OM, because of its non-pathologic  
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33 nature.  
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43 Cortactin is one of the molecules responsible for actin polymerization, a  
44 pivotal event related to invadopodia formation.<sup>14,22,23</sup> Our results showed that  
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46 cortactin was distributed in KOT neoplastic cells cytoplasm and mostly outlining the  
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48 cell edges. Those finding were expected, since cortactin concentrates at the cell  
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50 membrane to initiate invadopodia assembly.<sup>19,24,25,32</sup> In addition, cortactin can bind to  
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52 actin-related molecules like N-WASp and Arp2/3, forming complexes that may  
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54 possibly function as bridges to Tks5 in the initial steps of invadopodia formation.<sup>20,33-</sup>  
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Scr-kinases Tks4 and Tks5, also have an important role in invadopodia formation acting as promoters of these structures.<sup>25,36-39</sup> Our results demonstrated that both Tks4 and Tks5 showed elevated cytoplasmic expression in KOT neoplastic cells. Both molecules have a N-terminal Phox (PX) and SH3 domains, which after tyrosine phosphorylation, promote invadopodia formation and function.<sup>25,37-39</sup> Tks5 regulates actin cytoskeleton and cell membrane remodeling.<sup>22,23,37</sup> Tks4, recruits MT1-MMP, leading to focal MMP activity and ECM degradation, which in turn results in invadopodia maturation and stabilization.<sup>19,22,25,38</sup>

Elevated MT1-MMP levels in KOT cells was localized to the neoplastic cells cytoplasm. MT1-MMP is a transmembrane metalloproteinase responsible for pericellular matrix degradation.<sup>14</sup> MT1-MMP inhibition does not impair invadopodia formation but inhibits matrix degradation, probably due to a failure in MT1-MMP-mediated MMPs 2 and 9 local release.<sup>14</sup> The proteolytic activity of KOT neoplastic cells is reinforced here; with an elevated expression of MT1-MMP, which is supported by our previous result that demonstrated high levels of MMP-9 in KOT cells.<sup>4</sup>

KOT is known to be an intraosseous uni- or multicystic neoplasm, which may present daughter lesions.<sup>3,8,10,39</sup> These daughter lesions occur sporadically for unknown reasons. Our hypothesis is that invadopodia may take part in this process because of its ability to promote focal invasion. Invadopodia pericellular activity could induce detachment of one or more neoplastic cells from the primary lesion, leading to the formation of daughter lesions, and this event would be supported by overexpression of invadopodia-related proteins demonstrated in this study.

Another possible consequence of invadopodia influence in KOT clinical behavior could be related to the irregular pattern of growth sometimes presented by

1 this tumor. As a cystic lesion, KOT growth should occur relatively regularly, similar  
2 to other odontogenic cysts like dentigerous or radicular cysts, but this is often not the  
3 case.<sup>40</sup> KOT usually grows as multi-cystic lesions with irregular borders.<sup>41</sup>  
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5 Radiological features of KOTs with irregular and poorly defined edges suggest a  
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7 distinct pattern of growth in different parts of the same neoplasm. Those localized  
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9 differences could be influenced by different invadopodia-related bone degradation in  
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11 distinctive areas of this neoplasm.  
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17 Finally, it is already known that epidermal growth factor (EGF) and epidermal  
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19 growth factor receptor (EGFR) induces invadopodia formation.<sup>7,15,17,35,40,42,43</sup> These  
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21 molecules are overexpressed in KOT in accordance with our previous results.<sup>4</sup>  
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23 Although the limitations do not allow us to conclude that invadopodia are present in  
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25 KOT, overexpression of their main associated proteins strongly suggests that they are.  
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27 This speculation remains to be further elucidated.  
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33 The formation and function of invadopodia are directly related to the presence  
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35 of two main proteins, cortactin and MT1-MMP, and are reinforced by high expression  
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37 of their inducers Tks5 and Tks4.<sup>14</sup> All four proteins were found to be overexpressed in  
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39 KOT and localized to the basal layer where the invasion mechanisms occurs.  
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41 Interestingly, inflammation had no influence on the expression of these proteins in  
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43 any of the samples. Our data suggest that invadopodia may be present and would  
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45 influence KOT clinical behavior.  
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**CONFLICT OF INTEREST STATEMENT**

We declare that we have no conflict of interest.



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## Figure Legends

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**Fig. 1** Cortactin immunostaining in KOT, CCOT and OM samples. Cortactin was found in the cytoplasm of epithelial basal cells and in the cells of the upper stratum (A). A strong staining was observed in the basal layer, predominantly outlining cell edges. CCOT also expressed cortactin in a similar pattern but with less intensity (B). The overall cortactin expression in the OM was low (C). Significant difference was found between these 3 tissues in basal and subbasal layers (D). Magnification 630X. Scale bars: 20 $\mu$ m. \*= $p < 0.05$ ; \*\*= $p < 0.01$ ; \*\*\*= $p < 0.001$ ; \*\*\*\*= $p < 0.0001$ .

**Fig. 2** MT1-MMP immunostaining in KOT, CCOT and OM samples. MT1-MMP was found in the cytoplasm of KOT cells (A). A more intense immunolocalization of this protein was found near to the basement membrane in the basal layer of KOT. Although CCOT also expresses MT1-MMP, this expression appears to be lower compared to KOT (B). The expression of MT1-MMP was weaker in OM samples (C). KOT immunoexpresses higher amount of MT1-MMP (D). Magnification 630X. Scale bars: 20 $\mu$ m. \*= $p < 0.05$ ; \*\*= $p < 0.01$ ; \*\*\*= $p < 0.001$ ; \*\*\*\*= $p < 0.0001$ .

**Fig. 3** TKs4 immunostaining in KOT, CCOT and OM samples. TKs4 was expressed in the cytoplasm of KOT with an higher intensity in the layer with a granular form (A). CCOT also expressed TKs4, but with a very low expression in the suprabasal layer (B). Tiny amount of Tks4 expression was found in OM samples (C). Statistical analysis showed differences between KOT and the other samples, but no differences between CCOT and the OM (D). Magnification 630X. Scale bars: 20 $\mu$ m. \*= $p < 0.05$ ; \*\*= $p < 0.01$ ; \*\*\*= $p < 0.001$ ; \*\*\*\*= $p < 0.0001$ .

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**Fig. 4** TKs5 immunostaining in KOT, CCOT and OM samples. Similar cytoplasmic granular pattern of TKs5 staining, with higher intensity in the basal layer was found in KOT (A). CCOT showed a similar pattern but with equivalent distribution between the basal and suprabasal layers (B). Immunoexpression of Tks5 was very low in the OM (C). Statistical analysis demonstrated differences in the basal and suprabasal layers of all tissues, with higher expression in KOT (D). Magnification 630X. Scale bars: 20  $\mu\text{m}$ .  $\ast=p<0.05$ ;  $\ast\ast=p<0.01$ ;  $\ast\ast\ast=p < 0.001$ ;  $\ast\ast\ast\ast=p< 0.0001$ .

**Fig 5** Comparison between Cortactin, TKs5, MT1-MMP and TKs4 immunostaining in the basal and suprabasal layers in the same samples. Statistical differences were observed with higher expression in the basal layer in KOT for all proteins (A). No differences were found between these two layers in CCOT (B). The OM also showed higher expression of these proteins in the basal membrane, except for Tks5. The overall expression of Src-kinases Tks4 and Tks 5 was very low (C). Significance:  $\ast=p<0.05$ ;  $\ast\ast=p<0.01$ ;  $\ast\ast\ast= p < 0.001$ ;  $\ast\ast\ast\ast= p< 0.0001$ .



Figure 1  
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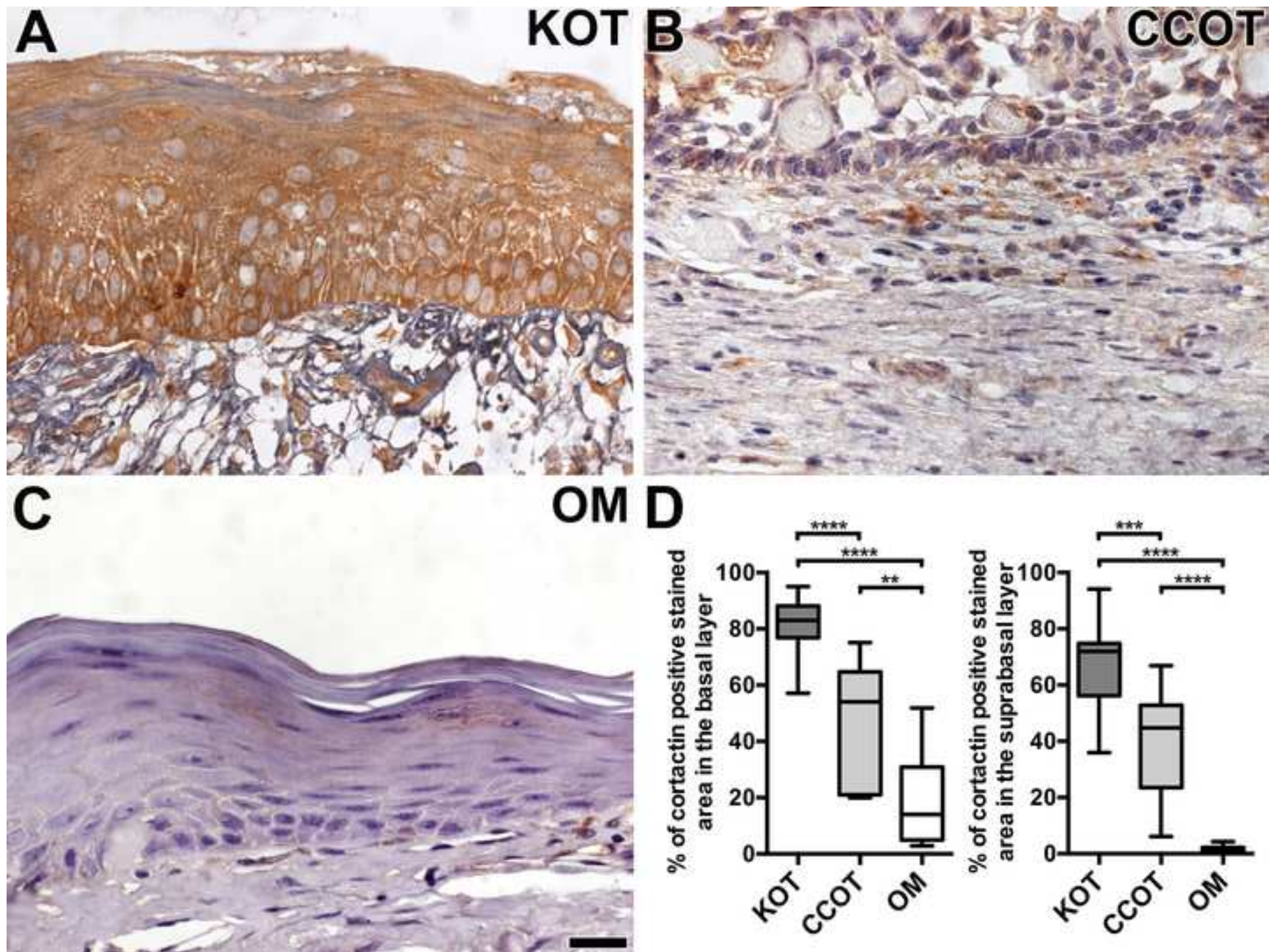


Figure 2  
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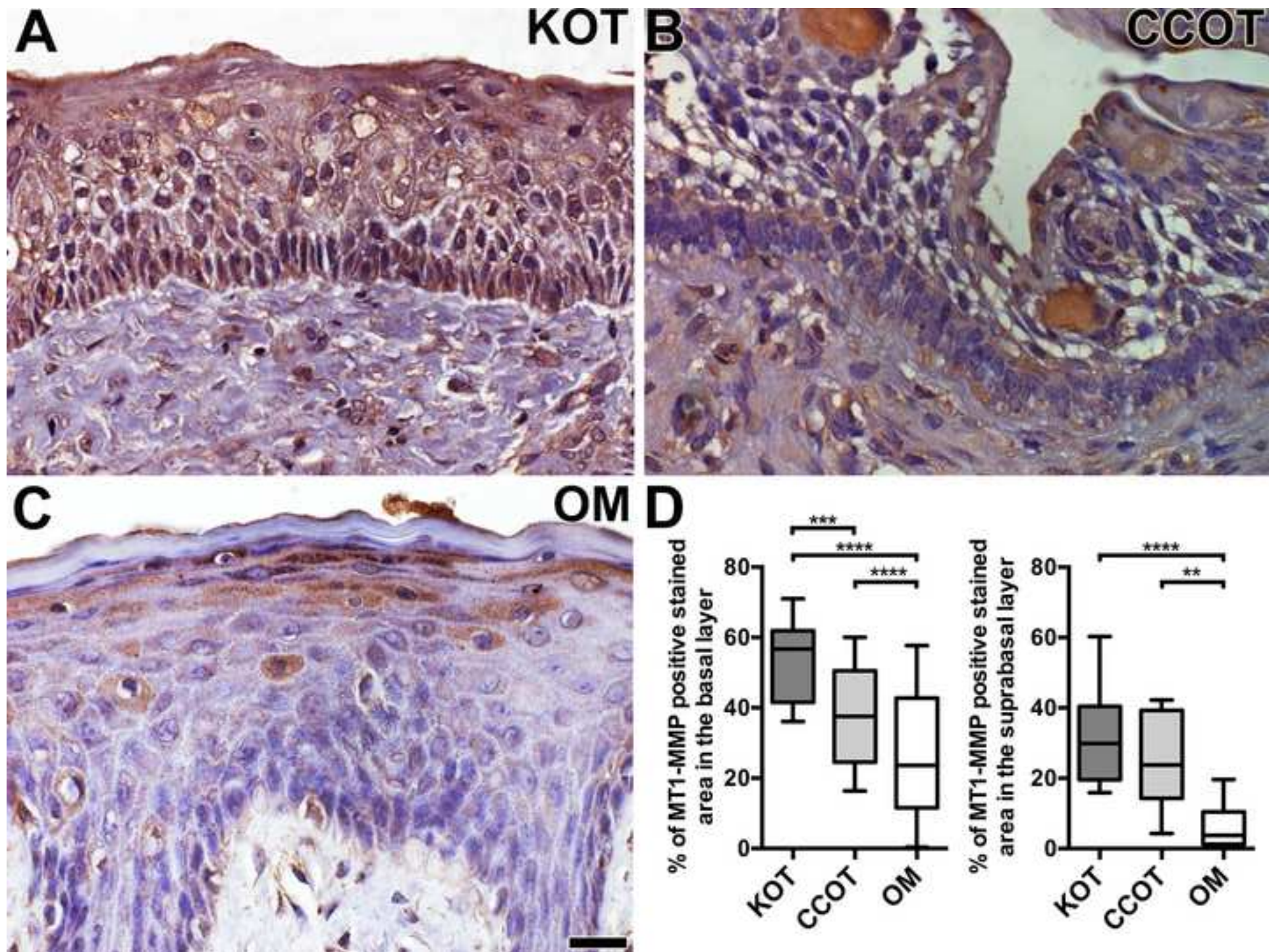


Figure 3  
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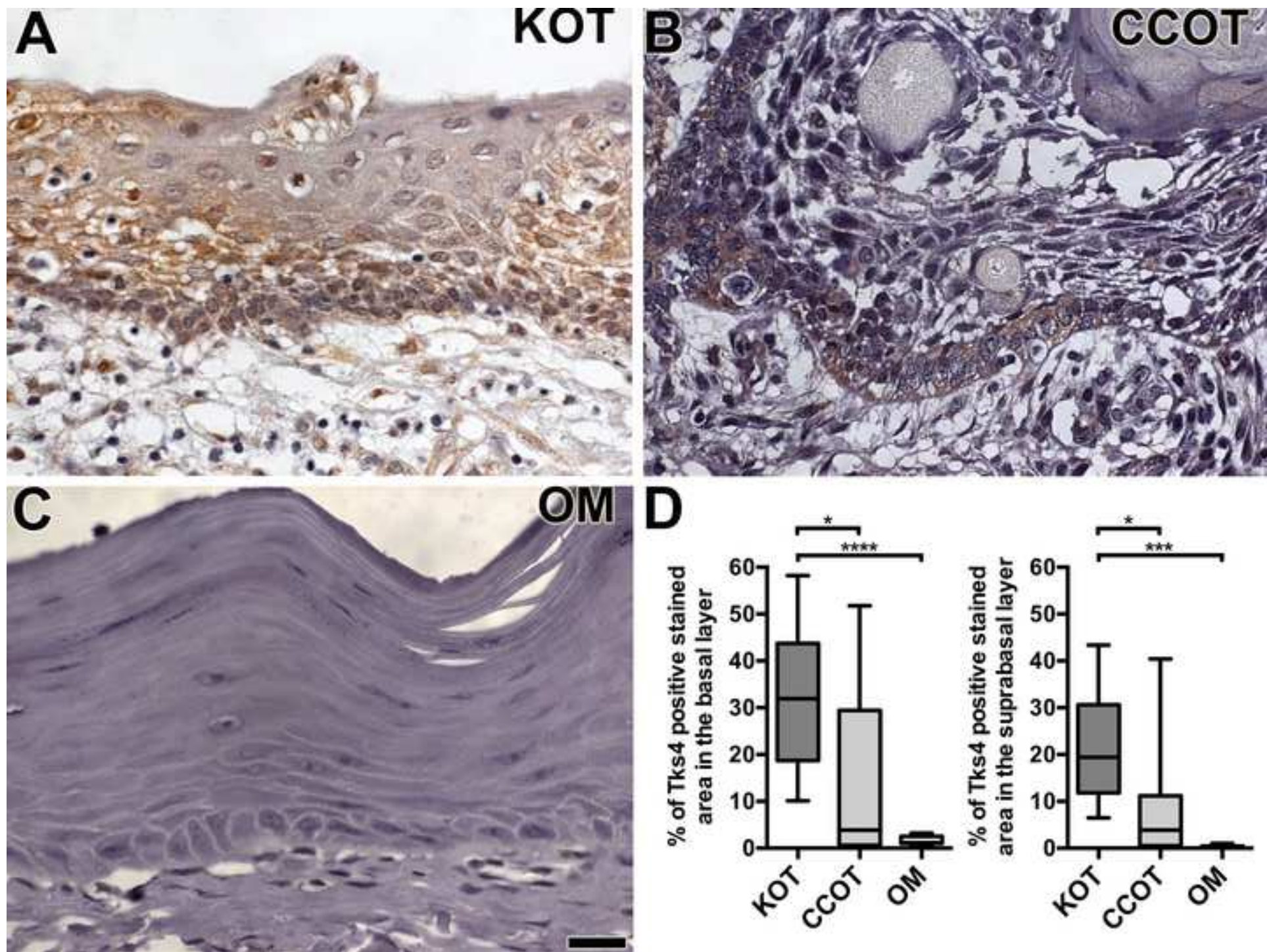


Figure 4  
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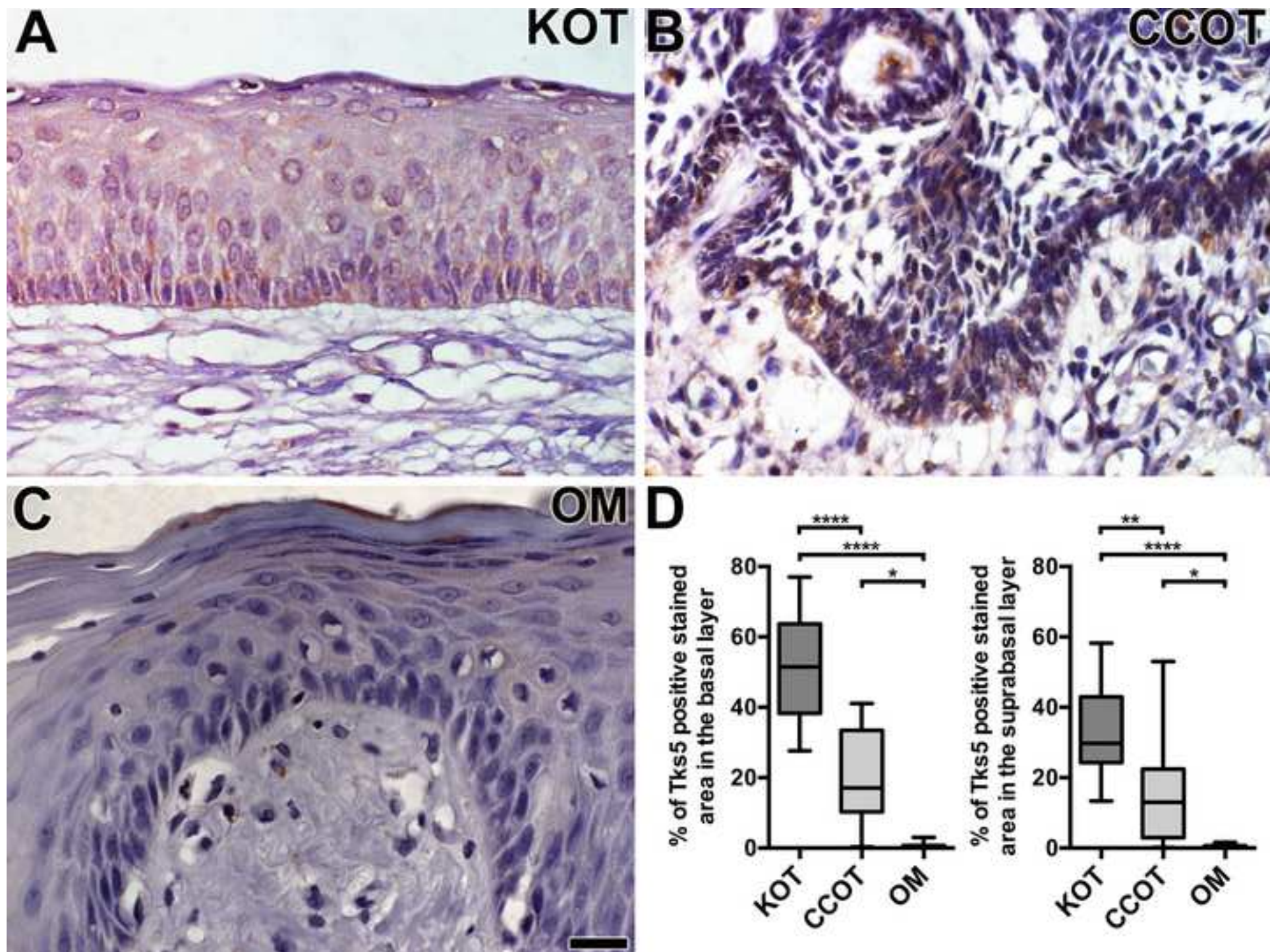
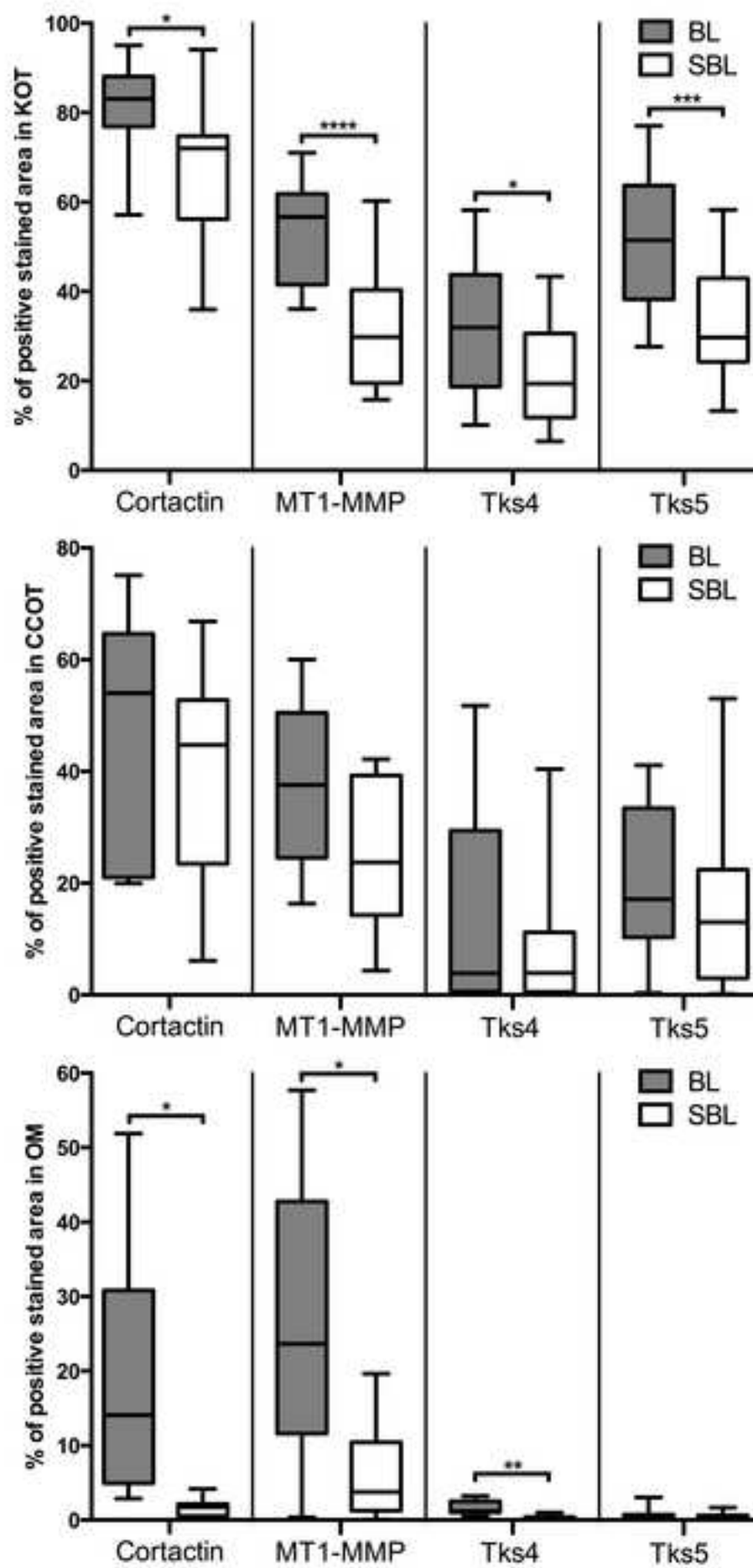


Figure 5

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**Table I:** Length of the basement membrane and inflammation assessment.

	Length of basement membrane (mm)				Inflammation grade				
	Mean	Min	Max	SD	0	1	2	3	A x B
KOT	9.220	8.029	11.484	0.834	-	8	4	4	ns
CCOT	9.759	8.026	15.213	1.513	-	2	3	3	ns
OM	13.537	8.006	23.451	3.904	-	5	2	1	ns

Abbreviation: KOT, keratocystic odontogenic tumor; CCOT, calcifying cystic odontogenic tumor; OM, oral mucosa; SD, standard deviation;

A x B describe the summary of statistical analysis for cortactin, membrane-type 1 matrix metalloproteinase, Tks4 and Tks5 between A, inflammation assessment Group A - grades 0-1 (mild-to-moderate) and B, inflammation assessment group B - grade 3 (intense); ns, non-significant;