Elsevier Editorial System(tm) for Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology
Manuscript Draft

Manuscript Number: TRIPLEO-D-16-00168R1

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

Article Type: Original Research Article

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

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 (max 40 words)

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

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

Authors' names:

André Luis Ribeiro Ribeiro[a,b] (double surname), DDS, MSc (Corresponding Author)

Natacha Malu Miranda da Costa[c], DDS

Adriane Sousa de Siqueira[d], DDS, MSc

Walessa Brasil da Silva[c] , DDS

Maria Sueli da Silva Kataoka[c], DDS, MSc, PhD

Ruy Gastaldoni Jaeger[e], DDS, MSc, PhD

Sérgio de Melo Alves-Junior[c], DDS, MSc, PhD

Andrew M. Smith[b], BSc, PhD

João de Jesus Viana Pinheiro[c], DDS, PhD

Author' institutions:

[a], Department of Oral and Maxillofacial Surgery, School of Dentistry, University Center of Pará - CESUPA, Belém, Brazil.

[b], Department of Microbial Diseases, Eastman Dental Institute, University College London, London, UK

[c], Cell Culture Laboratory, Faculty of Dentistry, Federal University of Pará. Rua Augusto Corrêa, 01, Guamá, 66075110, Belém, PA, Brazil.

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

[e], Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

Corresponding Author:

André Luis Ribeiro Ribeiro

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

Postal code: 66013 090

Phone number: 44 7823612356

Email: ribeiroalr@ig.com.br

Manuscript details

Total word count: 2021 words

Abstract word count: 149 words

Number of figures: 6

Number of tables: none

Number of references: 40

Number of supplementary: none

DISCLOSURES

Source of funding for research

We have read the operating principles and potential conflict of interest. **As it pertains to the article listed above, we declare that:**
We declare no sources of funding for this research, either current or within the past five years.

Financial interest disclosure

We have read the operating principles and potential conflict of interest. **As it pertains to the article listed above, we declare that:**
We declare no financial interest in this work, either current or within the past five years.

We state that the manuscript, or any part of it, has not been submitted or published and will not be submitted elsewhere for publication while being considered by the journal OOOO. We also disclose any prior presentation of abstracts at meetings and posting any part or all of the content on a website, even in draft form. We state that there isn't any poster presentations and meeting abstracts considered duplicate publication.

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

Keratocystic odontogenic tumor (KOT) is a benign odontogenic epithelial neoplasm with aggressive clinical behavior.[1-4] The local aggressiveness of KOT has been related to many characteristics, including the biological behavior of its neoplastic epithelial cells,[4] and genetic and epigenetic alterations[3,5]. For instance, studies have found evidence of allelic loss mainly in p16, p53, PTCH, MCC, TSLC1, LTAS2 and FHIT genes in KOT lesions.[3,5] Some studies have attempted to identify the role of different molecules that can influence the clinical behavior of odontogenic tumors,[4,6,7] however a full understanding regarding the mechanism related to KOT pathological behavior remains unknown.

KOT is characterized by a well-defined basal layer of columnar or cuboidal cells, with intense nuclear basophilia with polarity inversion. A thin capsule of connective tissue with few inflammatory cells and layers of parakeratin can also be found.[2,4,8,9] The basal layer sometimes shows a bud-shaped proliferation that may lead to the formation of daughter lesions.[4,8-12] KOT recurrence is a clinical challenge and occurs in nearly 23% of cases.[2] This high recurrence rate may be due to an increased proliferative activity observed in KOT, revealed by overexpression of proliferation markers such as Ki67 and proliferating cell nuclear antigen (PCNA) in KOT compared to other odontogenic cysts and tumors.[3,8]

A previous study has demonstrated the overexpression of matrix metalloproteinase-9 (MMP-9), epidermal growth factor (EGF) and tumor growth factor-alpha (TGF-α) in KOT cells.[4] Thus, it was inferred that elevated MMP-9, EGF and TGF-α levels could promote ECM degradation and release of more growth factors, contributing to KOT aggressiveness.[4] However, the mechanism of focal invasion of this tumor remains unknown.

Neoplastic cells invasion involves the dissemination of tumor cells from a primary site to the surrounding tissues.[13] Among the mechanisms that mediate this process, invadopodia formation and its proteolytic activity over ECM molecules are of great importance.[14,15] Invadopodia are actin-rich membrane protrusions that arise from the ventral surface of neoplastic cells and are vertically extended towards ECM.[16-18] It is known that different proteins are related to invadopodia formation and activity.[19] 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.[14,19-23]

Cortactin is responsible for the regulation of actin polimerization and is involved in the initial steps of invadopodia formation.[24,25] MT1-MMP is a transmembrane metalloprotease that plays a major role in the extracellular matrix remodeling and is strongly associated with neoplastic growth.[18] 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.[25] Tks5 has an important role in invadopodia elongation and is associated to cortactin.[26,27] Tks4, in turn, is responsible for regulating MT1–MMP localization, secretion and stabilization,[21,25] promoting localized ECM proteolysis and mediating tumor invasion.[14]

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.[20,27,28] High levels of cortactin and MT1-MMP have already been

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

MATERIALS AND METHODS

SAMPLE SELECTION AND CLASSIFICATION

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

IMMUNOHISTOCHEMISTRY

Formalin-fixed, paraffin-embedded tissues were studied by immunohistochemistry. Three-micron sections were obtained and mounted on poly-D-lysine-coated slides (Sigma Chemical Corp., St Louis MO, USA). Sections were dewaxed in xylene and rehydrated in graded ethanol. Antigen retrieval was carried out in Pascal chambers (Dako, Carpinteria, CA, USA) for 30 seconds. Sections were immersed in 3% H_2O_2 in methanol for 20 minutes for the inhibition of endogenous peroxidase activity and then blocked with 1% bovine serum albumin (BSA, Sigma®) in phosphate-buffered saline (PBS) for 1 hour. The slides were incubated with primary antibodies anti-cortactin (1:200, rabbit, Santa Cruz Biotechnology, CA, USA), anti-Tks5 (1:100, goat, Abcam, Cambridge, MA, USA), anti-Tks4 (1:100, rabbit, Abcam) and anti-MT1-MMP (1:50, mouse, R&D systems, Minneapolis, MN, USA). All primary antibodies were diluted in antibody diluent solution (Dako®) and incubated for 1 hour at room temperature. Subsequently, sections were incubated for 30 minutes with the biotin-free horseradish peroxidase (HRP)-labeled polymer of the LSAB Detection System (Dako®). Diaminobenzidine (Sigma®) was used as a chromogen, and the sections were counterstained with Mayer's hematoxylin (Sigma®). Replacement of specific primary antibodies with non-immune sera served as negative control and samples of oral squamous cell carcinoma were used as positive control (data not shown).

INFLAMMATORY AND IMMUNOSTAINING EVALUATION

Brightfield images from ten randomly selected images from each sample were acquired using an Axioskop 40 microscope (Carl Zeiss, Germany) equipped with a CCD color camera (AxiocCam MRc, Carl Zeiss). All images were acquired at the same magnification (400x). The basement membrane was measured using the

freehand line tool of the software ImageJ (public domain software developed by Wayne Rasband - NIMH, NIH, Bethesda, MD, USA, http://rsbweb.nih.gov/ij/). The inflammation score was assessed by counting the total number of inflammatory cells adjacent to the epithelium in all 10 images. Inflammation assessment was carried out in grades, being Grade 0 - no inflammation, Grade 1 - <15 cells/field, Grade 2 - 15-50 cells/field, and Grade 3 - >50 cells/field. The inflammatory score was calculated as the average of all fields examined. Samples of KOT, CCOT and OM were divided into two groups according to the inflammatory score: group A - grades 0-2 (mild-to-moderate) and group B - grade 3 (intense).[31] Comparison of the inflammation grades between group A and B and the immunoexpression of cortactin, MT1-MMP, Tks4, and Tks5, in basal and suprabasal layers were conducted for each tissue.

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

STATISTICAL ANALYSIS

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

comparison test. The parametric unpaired two-tailed t-test was used to analyze the differences between the basal and suprabasal layers. All tests were set to 95% confidence interval.

RESULTS

The length of the basement membrane varied from 8.01 mm to 23.45 mm, with the mean length of 9.22 mm in KOT, 9.76 mm in CCOT and 13.54 mm in OM. No cases presented inflammation grade 0, with inflammatory cells found in all samples. The summary of these results can be seen in Table I.

KOT and CCOT immunoexpress cortactin, MT1-MMP, Tks4 and Tks5

Cortactin, MT1-MMP, Tks4, and Tks5 were expressed in all KOT and CCOT samples. The oral mucosa showed low expression of cortactin, MT1-MMP and very low expression of Tks4 and Tks5. KOT neoplastic cells showed cytoplasmic expression of cortactin as dots in the epithelial basal and suprabasal layers. (Fig 1A). CCOT also expressed cortactin with a similar immunolocalization (Fig 1B). Small blood vessels and inflammatory cells were also positively stained for cortactin. Expression of this protein was not constant in the OM and usually found in the basal layer of samples (Fig 1C). Comparative analyze showed differences between the three groups, with the highest expression found in KOT, followed by CCOT and OM (Fig. 1D).

Cytoplasmic staining was also observed for MT1-MMP in KOT and CCOT neoplastic cells (Fig 2A-B). A weak cytoplasmic staining of MT1-MMP was found in the OM, which was reduced compared to both odontogenic tumors (2C). Inflammatory and endothelial cells were found to express MT1-MMP, specially in KOT and CCOT. MT1-MMP immunolocalization appeared to be more intense near to

the basement membrane in KOT. No difference was observed in the suprabasal layer between KOT and CCOT (2D).

Tks4 was immunostained with a granular pattern in the cytoplasm of KOT and CCOT (Fig. 3A-B). Tks4 showed a more intense expression in the basal layer in KOT and a discrete presence in the upper cells of the suprabasal layer in CCOT (Fig. 3A-B). Stromal cells expressed TKs4, which was poorly or even non-expressed in OM samples (Fig. 3C). Statistical analysis showed difference between KOT and the other samples in basal and suprabasal layers, but no difference was detected between CCOT and OM (Fig. 3D).

Similar to all the other proteins studied here, a cytoplasmic granular pattern of staining was found for Tks5. In KOT, Tks5 immunostaining was more intense in the basal layer and constantly expressed in all samples (Fig. 4A). The expression of Tks5 in CCOT was low, specially in the suprabasal layer (Fig. 4B). Expression of this protein in OM was very low (Fig. 4C). Statistical deference was found between the three tissues in both layers (Fig. 4D).

Although the CCOT samples also expressed cortactin, MT1-MMP, Tks4, and Tks5, the overall expression of these proteins were reduced in CCOT and OM compared to KOT.

In order to evaluate if there are differences between the expression of invadopodia-related proteins in the basal and suprabasal layer, a comparison of the expression of all proteins between these two layers was conducted. In comparison to OM tissue cortactin, MT1-MMP, Tks4, and Tks5 were all overexpressed in the basal layer of KOT samples, while no differences were observed in CCOT (Fig. 5). The potential role of inflammation in the expression of cortactin, MT1-MMP, Tks4 and

Tks5 was assessed by comparing the basal and suprabasal layers of group A (mild-to-moderate) and group B (intense) for each tissue. The level of tissue inflammation had no affect on the expression levels of cortactin, MT1-MMP, Tks4, and Tks5 in any tissue. These results are summarized in Table I.

DISCUSSION

Increased expression of cortactin and MT1-MMP has been related to aggressive behavior of various malignancies.[14,15] These proteins participate in invadopodia assembly. Invadopodia are membrane protrusions that allow neoplastic cells promote focal invasion. Membrane protrusions are driven by cortactin, while MT1-MMP is a key molecule for pericellular focal proteolysis. Cortactin and MT1-MMP are regulated by the Scr-kinases, Tks5 and Tks4 respectively. Our results showed overexpression of invadopodia-related proteins cortactin, MT1-MMP, Tks4, and Tks5 in KOT compared to CCOT and OM. All proteins were only expressed in the cytoplasm, with an increased expression in the basal layer of KOT samples, which strongly suggest the formation of invadopodia in this lesion. Low or no expression of invadopodia-related proteins was expected in OM, because of its non-pathologic nature.

Cortactin is one of the molecules responsible for actin polymerization, a pivotal event related to invadopodia formation.[14,22,23] Our results showed that cortactin was distributed in KOT neoplastic cells cytoplasm and mostly outlining the cell edges. Those finding were expected, since cortactin concentrates at the cell membrane to initiate invadopodia assembly.[19,24,25,32] In addition, cortactin can bind to actin-related molecules like N-WASp and Arp2/3, forming complexes that may possibly function as bridges to Tks5 in the initial steps of invadopodia formation.[20,33-35]

Scr-kinases Tks4 and Tks5, also have an important role in invadopodia formation acting as promoters of these structures.[25,36-39] 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.[25,37-39] Tks5 regulates actin cytoskeleton and cell membrane remodeling.[22,23,37] Tks4, recruits MT1-MMP, leading to focal MMP activity and ECM degradation, which in turn results in invadopodia maturation and stabilization.[19,22,25,38]

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.[14] 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.[14] 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.[4]

KOT is known to be an intraosseous uni- or multicystic neoplasm, which may present daughter lesions.[3,8,10,39] 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

this tumor. As a cystic lesion, KOT growth should occur relatively regularly, similar to other odontogenic cysts like dentigerous or radicular cysts, but this is often not the case.[40] KOT usually grows as multi-cystic lesions with irregular borders.[41] Radiological features of KOTs with irregular and poorly defined edges suggest a distinct pattern of growth in different parts of the same neoplasm. Those localized differences could be influenced by different invadopodia-related bone degradation in distinctive areas of this neoplasm.

Finally, it is already known that epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR) induces invadopodia formation.[7,15,17,35,40,42,43] These molecules are overexpressed in KOT in accordance with our previous results.[4] Although the limitations do not allow us to conclude that invadopodia are present in KOT, overexpression of their main associated proteins strongly suggests that they are. This speculation remains to be further elucidated.

The formation and function of invadopodia are directly related to the presence of two main proteins, cortactin and MT1-MMP, and are reinforced by high expression of their inducers Tks5 and Tks4.[14] All four proteins were found to be overexpressed in KOT and localized to the basal layer were the invasion mechanisms occurs. Interestingly, inflammation had no influence on the expression of these proteins in any of the samples. Our data suggest that invadopodia may be present and would influence KOT clinical behavior.

ACKNOWLEDGEMENTS

The author Andre Luis Ribeiro Ribeiro is grateful to CAPES foundation, Ministry of Education of Brazil, for funding his scholarship (grant no. 0698130).

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

REFERENCES

1. Shear M (2002) The aggressive nature of the odontogenic keratocyst: is it a benign cystic neoplasm? Part 1. Clinical and early experimental evidence of aggressive behaviour. Oral Oncol 38:219-226. doi: http://dx.doi.org/10.1016/S1368-8375(01)00065-3

2. Barnes L, Eveson JW, Reichart P, Sidransky D. World Health Organization Classification of Tumors. Pathology and genetics of head and neck tumours, 9th edn. Lion: International Agency for Research on Cancer Press, 2005; 296-300.

3. Gomes CC, Diniz MG, Gomez RS (2009) Review of the molecular pathogenesis of the odontogenic keratocyst. Oral Oncol 45:1011-1014. doi: 10.1016/j.oraloncology.2009.08.003.

4. Ribeiro ALR, Nobre RM, Alves-Junior SM, Kataoka MS, Barroso RF, Jaeger RG, Pinheiro JJV (2012). Matrix metalloproteinases, tissue inhibitors of metalloproteinases, and growth factors regulate the aggressiveness and proliferative activity of keratocystic odontogenic tumors. Oral Surg Oral Med Oral Pathol Oral Radiol 114:487-496. doi: 10.1016/j.oooo.2012.06.011.

5. Shear M (2002) The aggressive nature of the odontogenic keratocyst: is it a benign cystic neoplasm? Part 2. Proliferation and genetic studies. Oral Oncol 38:323-331. doi: http://dx.doi.org/10.1016/S1368-8375(01)00066-5

6. Ribeiro ALR, Nobre RM, Rocha GC, Lobato IHS, Alves-Junior SM, Jaeger RG, Pinheiro JJV (2011) Expression of metallothionein in ameloblastoma. A regulatory molecule? J Oral Pathol Med 40:516-519. doi: 10.1111/j.1600-0714.2011.01025.x

7. Siqueira AS, Carvalho MR, Monteiro AC, Freitas VM, Jaeger RG, Pinheiro JJV (2010) Matrix metalloproteinases, TIMPs and growth factors regulating

ameloblastoma behaviour. Histopathology 57:128-137. doi: 10.1111/j.1365-2559.2010.03596.x.

8. Mendes RA, Carvalho JF, van der Waal I (2010) Characterization and management of the keratocystic odontogenic tumor in relation to its histopathological and biological features. Oral Oncol 46:219-225. doi: 10.1016/j.oraloncology.2010.01.012.

9. Guler N, Sencift K, Demirkol O (2012) Conservative management of keratocystic odontogenic tumors of jaws. ScientificWorldJournal 2012:680397. doi: 10.1100/2012/680397.

10. Kaczmarzyk T, Mojsa I, Stypulkowska J (2012) A systematic review of the recurrence rate for keratocystic odontogenic tumour in relation to treatment modalities. Int J Oral Maxillofac Surg 41:756-767. doi: 10.1016/j.ijom.2012.02.008.

11. Kuroyanagi N, Sakuma H, Miyabe S et al (2009) Prognostic factors for keratocystic odontogenic tumor (odontogenic keratocyst): analysis of clinico-pathologic and immunohistochemical findings in cysts treated by enucleation. J Oral Pathol Med 38:386-392. doi: 10.1111/j.1600-0714.2008.00729.x.

12. Simiyu BN, Butt F, Dimba EA, Wagaiyu EG, Awange DO, Guthua SW, lootweg PJ (2013) Keratocystic odontogenic tumours of the jaws and associated pathologies: a 10-year clinicopathologic audit in a referral teaching hospital in Kenya. J Craniomaxillofac Surg 41:230-234. doi: 10.1016/j.jcms.2012.09.006.

13. Grunert S, Jechling M, Beug H (2003) Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. Nat Rev Mol Cell Biol 4:657-665. doi:10.1038/nrm1175

14. Artym VV, Zhang Y, Seillier-Moiseiwitsch F, Yamada KM, Mueller SC (2006) Dynamic interactions of cortactin and membrane type 1 matrix

metalloproteinase at invadopodia: defining the stages of invadopodia formation and function. Cancer Res 66:3034-3043. doi: 10.1158/0008-5472.CAN-05-2177

15. Yamaguchi H (2012) Pathological roles of invadopodia in cancer invasion and metastasis. Eur J Cell Biol 91:902-907. doi: 10.1016/j.ejcb.2012.04.005.

16. Nascimento CF, Gama-De-Souza LN, Freitas VM, Jaeger RG (2010) Role of MMP9 on invadopodia formation in cells from adenoid cystic carcinoma. Study by laser scanning confocal microscopy. Microsc Res Tech 73:99-108. doi: 10.1002/jemt.20761.

17. Nascimento CF, de Siqueira AS, Pinheiro JJ, Freitas VM, Jaeger RG (2011) Laminin-111 derived peptides AG73 and C16 regulate invadopodia activity of a human adenoid cystic carcinoma cell line. Exp Cell Res 317:2562-2572. doi: 10.1016/j.yexcr.2011.08.022

18. Pinheiro JJ, Nascimento CF, Freitas VM, de Siqueira AS, Junior SM, Jaeger RG (2011) Invadopodia proteins, cortactin and membrane type I matrix metalloproteinase (MT1-MMP) are expressed in ameloblastoma. Histopathology 59:1266-1269. doi: 10.1111/j.1365-2559.2011.03995.x.

19. Murphy DA, Courtneidge SA (2011). The 'ins' and 'outs' of podosomes and invadopodia: characteristics, formation and function. Nat Rev Mol Cell Biol 12:413-426. doi: 10.1038/nrm3141.

20. Yamaguchi H, Oikawa T (2010) Membrane lipids in invadopodia and podosomes: key structures for cancer invasion and metastasis. Oncotarget 1:320-328.

21. Buccione R, Caldieri G, Ayala I (2009) Invadopodia: specialized tumor cell structures for the focal degradation of the extracellular matrix. Cancer Metastasis Rev 28:137 -149. doi: 10.1007/s10555-008-9176-1.

22. Clark ES, Weaver AM (2008) A new role for cortactin in invadopodia: regulation of protease secretion. Eur J Cell Biol 87:581-590. doi: 10.1016/j.ejcb.2008.01.008.

23. Monsky WL, Chen WT (1993) Proteases of cell adhesion proteins in cancer. Semin. Cancer Biol 4:251-258.

24. Burger KL, Learman BS, Boucherle AK et al (2014) Src -dependent Tks5 phosphorylation regulates invadopodia-associated invasion in prostate cancer cells. Prostate 74:134-148. doi: 10.1002/pros.22735.

25. Courtneidge SA (2012) Cell migration and invasion in human disease: the Tks adaptor proteins. Biochem Soc Trans 40:129-132. Doi: 10.1042/BST20110685.

26. Crimaldi L, Courtneidge SA, Gimona M (2009) Tks5 recruits AFAP -110, p190RhoGAP, and cortactin for podosome formation. Exp Cell Res 315:2581-2592. doi: 10.1016/j.yexcr.2009.06.012.

27. Sharma VP, Eddy R, Entenberg D, Kai M, Gertler FB, Condeelis J (2013) Tks5 and SHIP2 regulate invadopodium maturation, but not initiation, in breast carcinoma cells. Curr Biol 23:2079-2089. doi: 10.1016/j.cub.2013.08.044.

28. Stylli SS, Kaye AH, Lock P (2008) Invadopodia: at the cutting edge of tumour invasion. J Clin Neurosci 15:725-737. doi: 10.1016/j.jocn.2008.03.003.

29. de Paula AM, Carvalhais JN, Domingues MG, Barreto DC, Mesquita RA (2000) Cell proliferation markers in the odontogenic keratocyst: effect of inflammation. J Oral Pathol Med 29(10):477-82. doi: 10.1034/j.1600-0714.2000.291001.x

30. Li TJ, Browne RM, Matthews JB (1994) Quantification of PCNA+ cells within odontogenic jaw cyst epithelium. J Oral Pathol Med 23(4):184-9. doi: 10.1111/j.1600-0714.1994.tb01110.x

31. Johann AC, Caldeira PC, Caliari MV, Gomez RS, Aguiar MC, Mesquita RA (2015) Metallothionein immunoexpression in non-syndromic and syndromic keratocystic odontogenic tumour. Med Oral Patol Oral Cir Bucal 1;20(4):e408-12. doi:10.4317/medoral.20418

32. Clark ES, Whigham AS, Yarbrough WG, Weaver AM (2007) Cortactin is an essential regulator of matrix metalloproteinase secretion and extracellular matrix degradation in invadopodia. Cancer Res 67:4227-4235. doi: 10.1158/0008-5472.CAN-06-3928

33. Desai B Ma T, Chellaiah MA (2008) Invadopodia and matrix degradation, a new property of prostate cancer cells during migration and invasion. J Biol Chem 283:13856 - 13866. doi: 10.1074/jbc.M709401200.

34. Fekete A, Bogel G, Pesti S, Peterfi Z, Geiszt M, Buday L (2013) EGF regulates tyrosine phosphorylation and membrane -translocation of the scaffold protein Tks5. J. Mol. Signal 8:8. doi: 10.1186/1750-2187-8-8.

35. Oser M, Yamaguchi H, Mader C et al (2009) Cortactin regulates cofilin and N-WASp activities to control the stages of invadopodium assembly and maturation. J Cell Biol 186:571 -587. doi: 10.1083/jcb.200812176.

36. Tarone G, Cirillo D, Giancotti FG, Comoglio PM, Marchisio PC (1985) Rous sarcoma virus -transformed fibroblasts adhere primarily at discrete protrusions of the ventral membrane called podosomes. Exp Cell Res 159:141-157

37. Blouw B, Seals DF, Pass I, Diaz B, Courtneidge SA (2008) A role for the podosome/invadopodia scaffold protein Tks5 in tumor growth in vivo. Eur J Cell Biol 87:555-567. doi: 10.1016/j.ejcb.2008.02.008.

38. Buschman, MD, Bromann PA, Cejudo-Martin P, Wen F, Pass I, Courtneidge SA (2009) The novel adaptor protein Tks4 (SH3PXD2B) is required for functional podosome formation. Mol Biol Cell 20:1302-1311. doi: 10.1091/mbc.E08-09-0949.

39. Johnson NR, Gannon OM, Savage NW, Batstone MD (2014) Frequency of odontogenic cysts and tumors: a systematic review. J Investig Clin Dent J 5:9-14. doi: 10.1111/jicd.12044.

40. Toller P (1967) Origin and growth of cysts of the jaws. Ann R Coll Surg Engl. 40:306-336.

41. Sansare K, Raghav M, Mupparapu M, Mundada N, Karjodkar FR, Bansal S, Desai R (2013) Keratocystic odontogenic tumor: systematic review with analysis of 72 additional cases from Mumbai, India. Oral Surg Oral Med Oral Pathol Oral Radiol 115:128-139. doi: 10.1016/j.oooo.2012.10.005.

42. Frittoli E, Palamidessi A, Disanza A, Scita G (2011) Secretory and endo/exocytic trafficking in invadopodia formation: the MT1-MMP paradigm. Eur J Cell Biol 90:108-114. doi: 10.1016/j.ejcb.2010.04.007

43. Hwang YS, Park KK, Chung WY (2012) Invadopodia formation in oral squamous cell carcinoma: the role of epidermal growth factor receptor signalling. Arch Oral Biol 57:335-43. doi: 10.1016/j.archoralbio.2011.08.019.

Figure Legends

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 subrabasal layers (D). Magnification 630X. Scale bars: 20µm. *=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: 20µm. *=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: 20µm. *=p<0.05; **=p<0.01; ***= p < 0.001; ****= p< 0.0001.

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 µm. *=p<0.05; **=p<0.01; ***= p < 0.001; ****= 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: *=p<0.05; **=p<0.01; ***= p < 0.001; ****= p< 0.0001.

Figure 1

Click here to download high resolution image

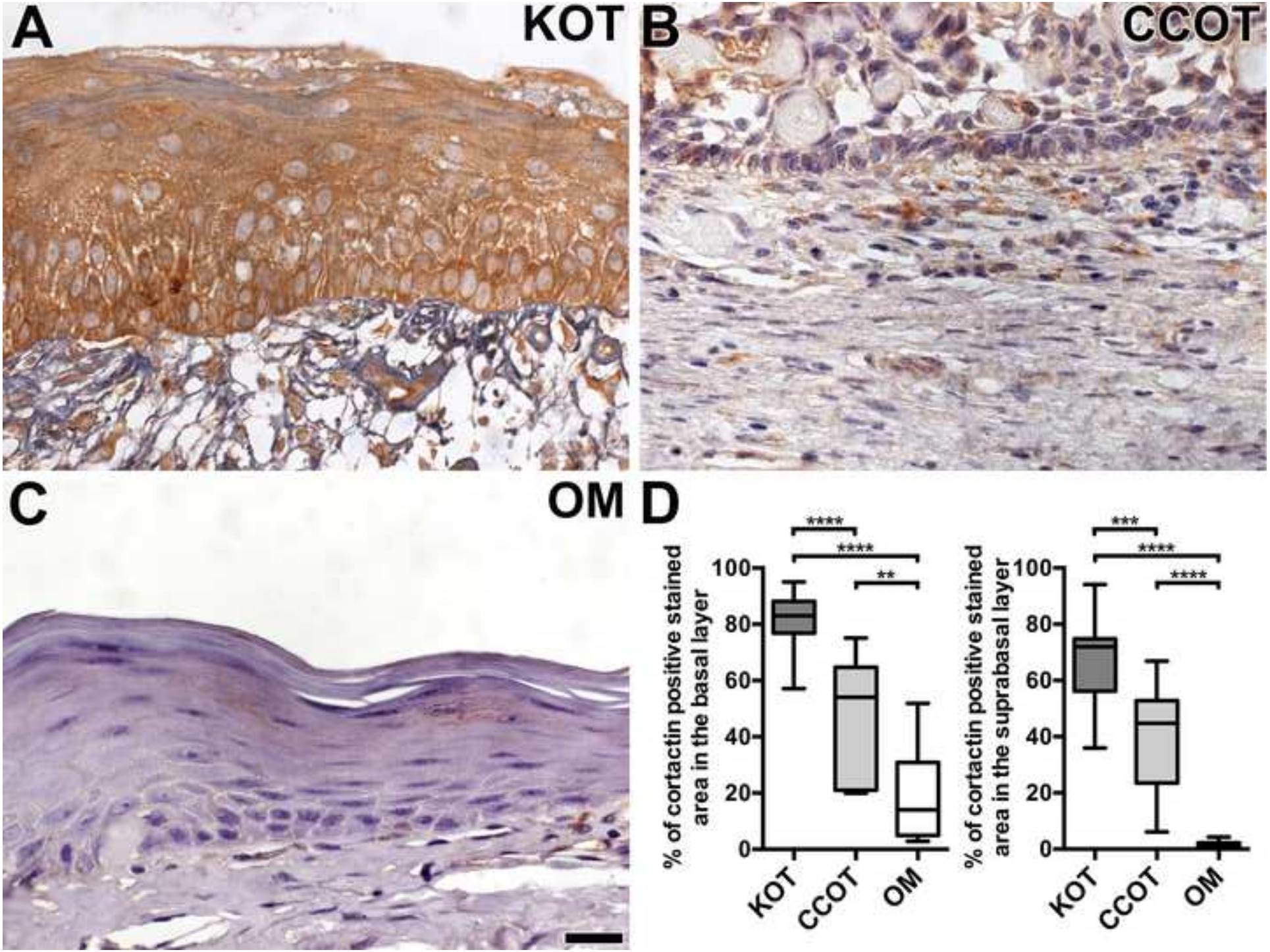

Figure 2

Click here to download high resolution image

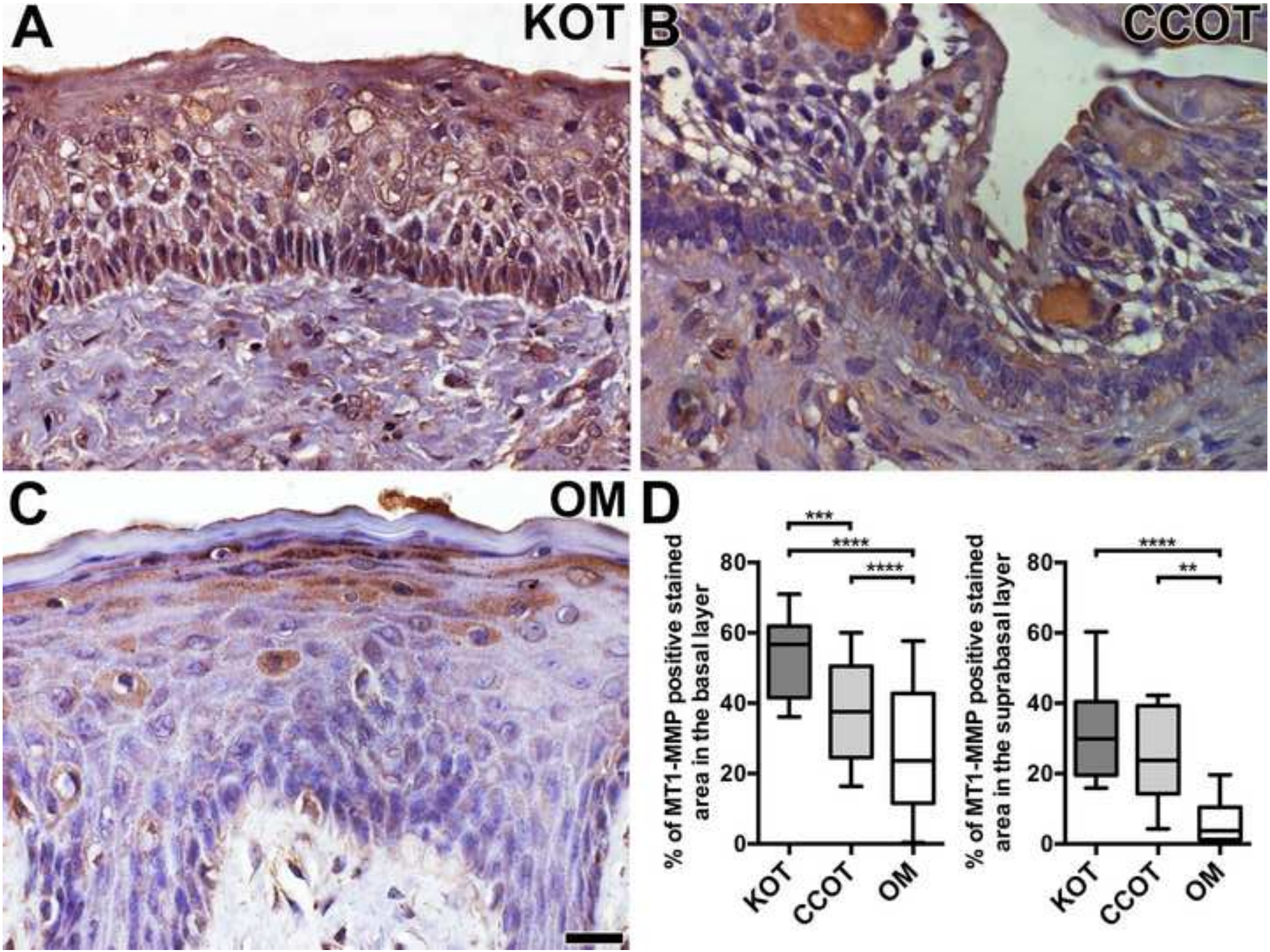

Figure 3

Click here to download high resolution image

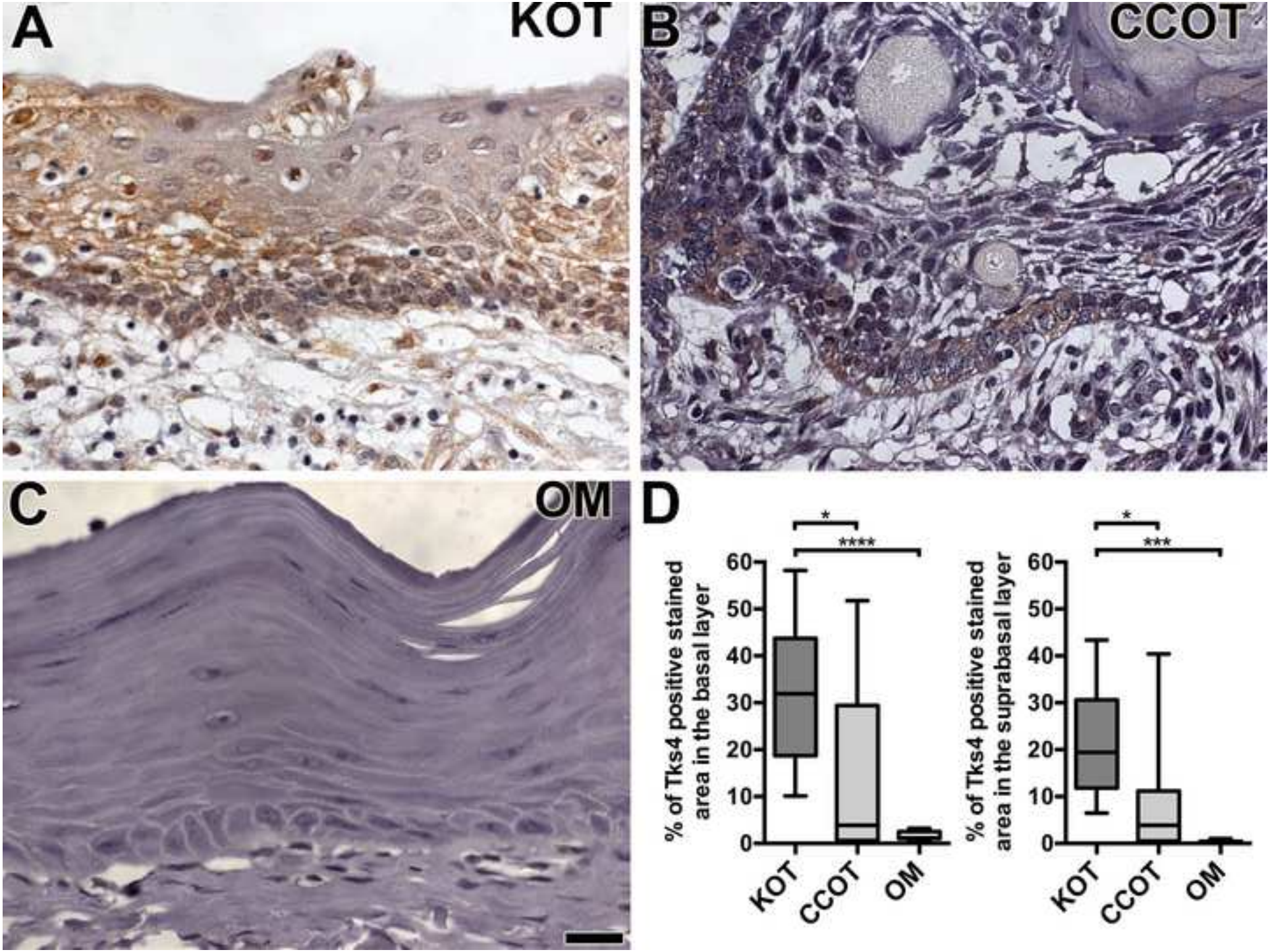

Figure 4

Click here to download high resolution image

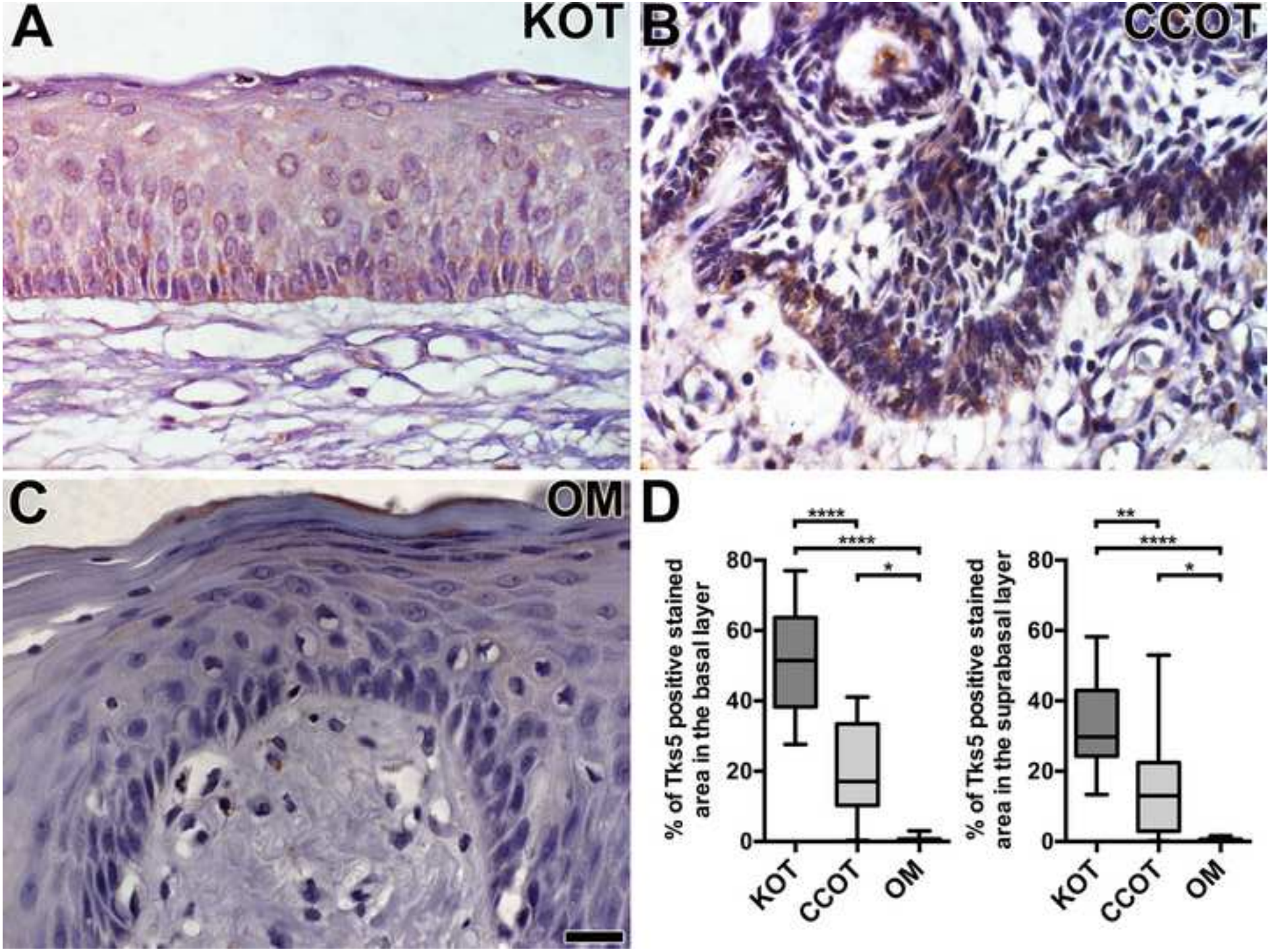

Figure 5

Click here to download high resolution image

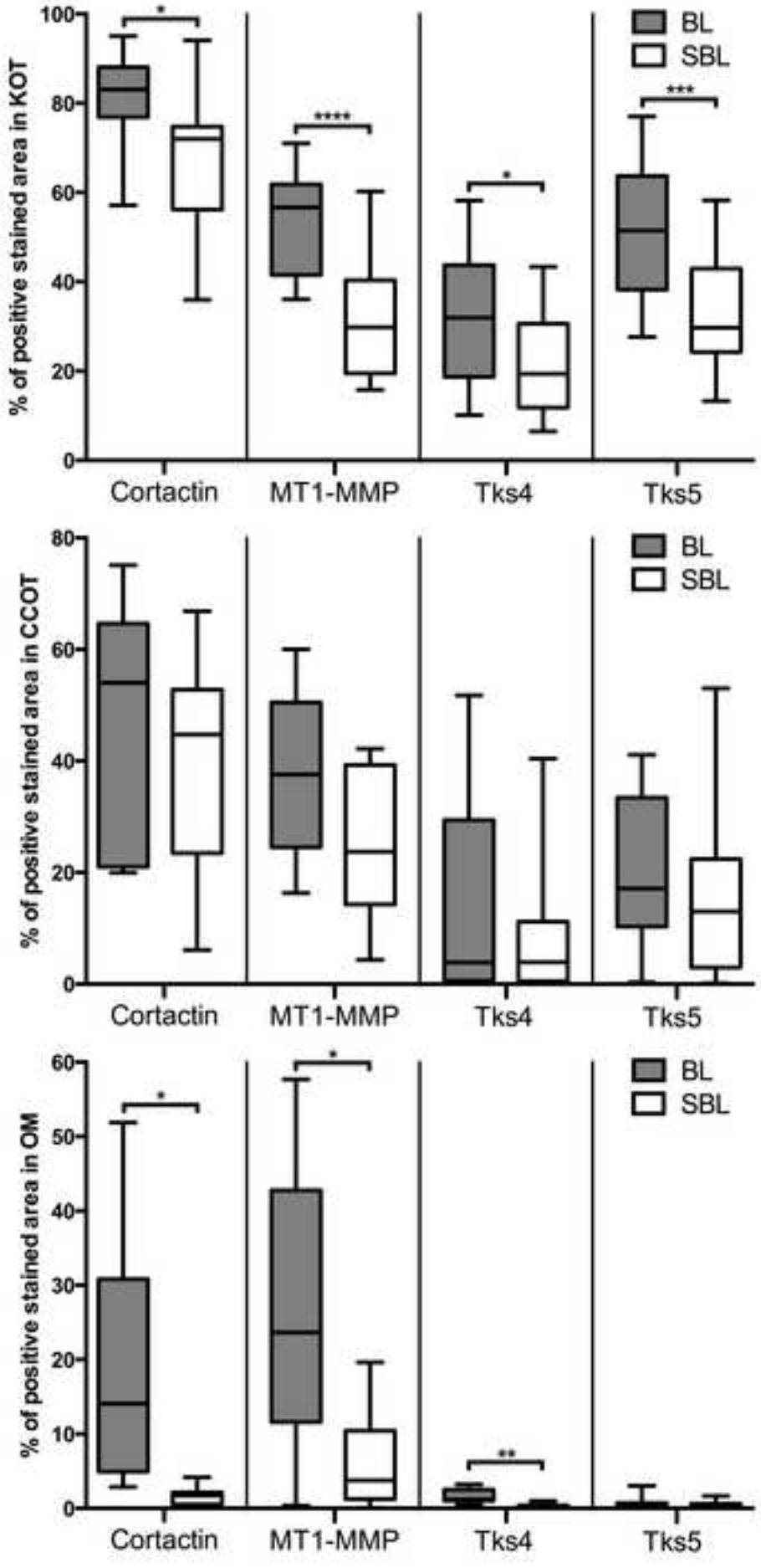

Table I: Length of the basement membrane and inflammation assessment.

	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;