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Molecular Assessment of Kallikrein 3 in Ameloblastoma and Odontogenic Keratocyst

Kholoud Ahmed
Oral pathology, faculty of dentistry, Ahram candian university, 6th of October city, Egypt,
kholoudrefaat@azhar.edu.eg

Mohamed El Sebie
Lecturer of medical biochemistry, Faculty of Medicine, Cairo University, Cairo, Egypt.,
mohamed_el_sebaie@hotmail.com

Nadia Radi
Associate Professor of Oral and Dental Medicine for Girls, Al-Azhar University, Cairo, Egypt.,
n_radi@yahoo.com

Amal Abd El Rahman
Professor of Oral and Maxillofacial Pathology, Faculty of Dentistry, Cairo University, Cairo, Egypt,
amalabdel.rahman2011@yahoo.com

Eman AboHager
Professor and Head of Oral and Dental pathology Department Faculty of Dental Medicine for Girls, Al-Azhar University, Cairo Egypt, emanhager@yahoo.com

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Molecular Assessment of Kallikrein 3 in Ameloblastoma and Odontogenic Keratocyst

Kholoud R. Ahmed [1*], Mohamed El Sebie [2], Nadia A. Radi [3], Amal H. Abd El Rahman [4], Eman A. AboHager [5]

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azhardentj@azhar.edu.eg

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ABSTRACT

Purpose: The current research was conducted to assess KLK3 at molecular and protein level in AB and OKC to shed more light on its role on biologic behaviour of these lesions. **Material and methods: 30** paraffin embedded blocks of AB, OKC and Dentigerous Cyst (DC) as a control were included. Immunohistochemical staining technique with KLK3 antibody was applied to all the tissue blocks and RT-PCR KLK3 gene were done for all specimens. **Results:** The mean area percent of KLK3 expression was significantly greater in AB and OKC compared with DC. According to the current study, presence of significant difference between AB and OKC in relation to control indicates that KLK3 antibody is related the aggressive biologic behaviour of AB and OKC. **Conclusion:** Increased expression of KLK3 protein and gene expression in AB and OKC suggests that KLK3 may have a role in the intrinsic and limitless growth potential of these lesions.

KEYWORDS

Ameloblastoma;
Odontogenic Keratocyst; KLK3.

INTRODUCTION

Odontogenic tumours (OTs) are a group of lesions characterised by different clinical behaviour and variable histopathologic spectrum from hamartomas to malignancy(1). OTs show inductive interactions between different odontogenic epithelium which forms the basis of their classification. OTs are mainly divided into benign and malignant

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1. Assistant Lecturer of Oral pathology Ahram Canadian University, Cairo, Egypt.
2. Lecturer of medical biochemistry, Faculty of Medicine, Cairo University, Cairo, Egypt.
3. Associate Professor of Oral and Dental Medicine for Girls, Al-Azhar University, Cairo, Egypt.
4. Professor of Oral and Maxillofacial Pathology, Faculty of Dentistry, Cairo University, Cairo, Egypt.
5. Professor and Head of Oral and Dental pathology Department Faculty of Dental Medicine for Girls, Al-Azhar University, Cairo Egypt.

* Corresponding author email: kholoudrefaat@azhar.edu.eg

tumours according to their biologic behaviour following head and neck tumours classification published in 2017 (WHO)[2]. Future studies following the new classification are expected to report decreased numbers of OTs which is attributed to the reclassification of some entities[3].

AB accounts for almost 14% of tumours and cysts that affect upper and lower jaws, and it is the most extensive OT in developing countries. It is the most encountered tumor rising from odontogenic epithelium, AB is characterized by being benign with a local invasive action and a high rate of recurrence[4]. The invasion of surrounding healthy tissues by tumor cells is one of the important criteria in tumor progression. Therefore, identification of invasive activities in ABs may be useful to predict their biological behaviour[5]. Also, the aggressive treatment might lead to a lower recurrence rate than conservative treatment[6]. AB has a tendency to insinuate between the cancellous bones due to its invasive action, partial maxillectomy or mandibulectomy with a safety margin of healthy bone is the best treatment option[7].

The most recent classification of WHO (2017) reclassified Para keratinized odontogenic keratocyst as a cyst not a tumor. Recent studies demonstrated that OKC behave clinically as non-neoplastic lesions and treated as cysts[8]. OKC is still the most difficult to be treated because of its local aggressive behaviour and high rate of recurrence. OKC was reclassified a neoplasm by WHO in 2005 and known as KCOT. OKC is characterised by destructive growth rate and high rate of recurrence. OKC grows in the anteroposterior direction and may reach large size without significantly damaging the jaws anatomy. It has a shallow fibrous capsule lined with Para keratinized crumbly squamous epithelium with high mitotic activity and its remnants have the tendency to be left on removal of the lesion contributing to the recurrence of the lesion[9,10].

The tumour invasive extension can be investigated by the expression and construction of several genes and proteins by the tumor cells. Yet, recent clinical parameters face many struggles to detect the neoplastic behaviour in both AB and OKC. Unusually, KLKs have a great role in allowing cancer progression, the cell-biological programming, especially through lysis of extracellular vital mediators such as cell to cell adhesion proteins, cytokines, proteins that are bounded to the membrane and receptors, ECM proteins and growth factors[11]. KLK 3 is known as prostate-specific antigen, it is the most investigated and best studied in the KLK family. So, KLKs have been categorised as biomarkers, that are categorised for their comparative excess disease conditions and examined for their action in human tissue physiology[12]. KLK 3 shows to be incorporated in the invasion process of the tumours by direct degradation of the ECM. also, has a role in triggering the tumours by transforming growth factor-β and IGF, It could be that KLK3 contributes to the growth rate of these lesions through the different mechanisms and signalling cascades or through another mechanisms that are still unknown[13]. The application of KLK3 clinically as a biomarker shed the light to the clinical importance of Kallikreins in the diagnosis and intensive care of tumours and their ability for recurrence. Studies shows that KLKs have main role in the spreading of cancer cells through the migration effect that could be done by KLKs in the cell and tissue. It is known that KLKs have proteolytic action toward ECM proteins, receptors assured to signalling, the cell membrane, cell adhesion proteins, growth factors[14].

RT-PCR accuracy is affected by several factors. These factors depend on the quality and quantity of mRNA templates, different reaction efficiency and the variation between cells or tissues in general transcriptional activity and mRNA half-life. These factors depend on gene regulation expression and expression of an internal control or gene referencing. The appearance of this gene should be continuous in the human tissues or the investigated cells and should be stable in response to new treatments[15].

MATERIAL AND METHODS

Case selection

Research Ethics Committee approval of Faculty of Dental Medicine for Girls Al Azhar University was obtained (REC-PA-21-03).

A total of 10 specimens from each lesion were repossessed from the archival paraffin blocks from the pathological files of the Department of Oral and Maxillofacial Pathology, Faculty of Oral and Dental Medicine, Department of General Pathology, Faculty of Medicine Cairo University and from Maxillofacial Surgery Department of Naser Institute.

Histological analysis

Using H&E stain for revaluation of the cases was carried out to confirm their diagnosis, and reclassifying them according to WHO[8].

Immunohistochemical analysis

Immunostaining for KLK3 was performed at National Cancer Institute using Ventana Benchmark auto Stainer (USA) and the following steps occurred automatically. The tissue sections undergo hydration and deparaffinization in a descending manner in alcohol for about 10 minutes. Boiling the tissue sections in pH 6.0 and 10mM citrate buffer for 10-20 minutes is mandatory before staining the formalin fixed tissue then allowed to cool for 20 minutes at room temperature (antigen retrieval step). Incubation of the sections was done in 0.3% hydrogen peroxide for 30 minutes; this step allows blocking the activity of endogenous peroxidase enzyme. Then washing the sections before applying 100 microns of KLK3 polyclonal antibody with (1:1400-800) dilution under 30°C incubation temperature and wait for 60 minutes, then the secondary antibody was applied for 30 minutes. Application of diaminobenzidine was done at room temperature for 5 minutes. with Haematoxylin stain applied as a counter stain for 8 minutes and as a post counter stain with bluing reagent for 4 minutes. Extraction of the Slides was done followed by racks arrangement. Washing the slides for 5 minutes in tap water then dehydration of alcohol for 5 minutes was done in each container in ascending manner. Clearing of the slides in xylene then application of the cover slips and mounting using Canada balsam agent was carried out[16].

RT- PCR analysis

First step entails wax removal then 25 microns from each paraffin block were cut and put in eppindorff, 500 µl of xylene were added to each sample and placed in vigorous shaker for 10 min, centrifuged for 3 min at 12.000, then distraction of flow-through was done. The previous steps were repeated for 3 times. Tissue lysis buffer with 200 µl dilution was added to each sample then centrifugation and distraction of flow-through were followed as above. Secondly, isolated RNA samples were moved into RNA binding column and collected in a collection tube followed by centrifugation at 10,000 for 1 min, then distraction of flow-through was applied. Adding 500 µl of Wash Buffer then centrifugation at maximum for 1 min, and then distraction of the flow-through was done. Seventy µl dilution of DNase I Digestion Mixture was pipetted into the RNA binding column and this mix was left for incubation for 15 min at room temperature followed by the addition of 500 µl of Inhibitor Removal Buffer then centrifuged at maximum for 1 min, and then the flow-through was discarded. 500 µl of wash Buffer 1 was added then centrifuged at 10,000 for 1 min, and then the flow-through was discarded. 500 µl of wash Buffer 2 was added then centrifuged at 10,000 for 1 min, and then the flow-through was discarded. The columns were then centrifuged at 10,000 for 1 min to remove traces of buffer. The columns were placed into micro centrifuge tube, then 40 µl of RNase- Free Water were directly added onto the membrane and stood for 1 min, then centrifuged at 10,000 for 1 min, finally RNA was kept at -20°C.

9-Spectrophotometer was used for the assessment of the concentration and sanitization of extracted nucleic acid (measurement of extracted nucleic acid using 260/280 absorbance UV)[17].

Statistical analysis

Coding of the data was done and submitted using the statistical package for the Social Sciences (SPSS) version 26 (IBM Corp., Armonk, NY, USA). Summarising the data using median, minimum, mean, standard deviation and maximum was done. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis test and adjusted Mann-Whitney test as post hoc test P-values less than 0.05 were considered as statistically significant.

RESULTS

Histopathological findings

The histopathologic examination of all DC cases showed epithelial lining contains thin layers of nonkeratinized flattened cells. Mucous cells appear in some focal areas in the epithelial lining of dentigerous cysts **Fig. (1A).** The histopathologic examination of all AB cases showed Solid multiple follicles, each follicle showed peripheral ameloblast like cells and central stellate reticulum like cells **Fig. (1B)**. The histopathologic examination of all OKC cases showed an even layer of stratified squamous epithelium, this layer composed of six to eight cells, the luminal surface showed flattened epithelial cells with Para keratin which exhibited corrugated appearance, the basal epithelial layer

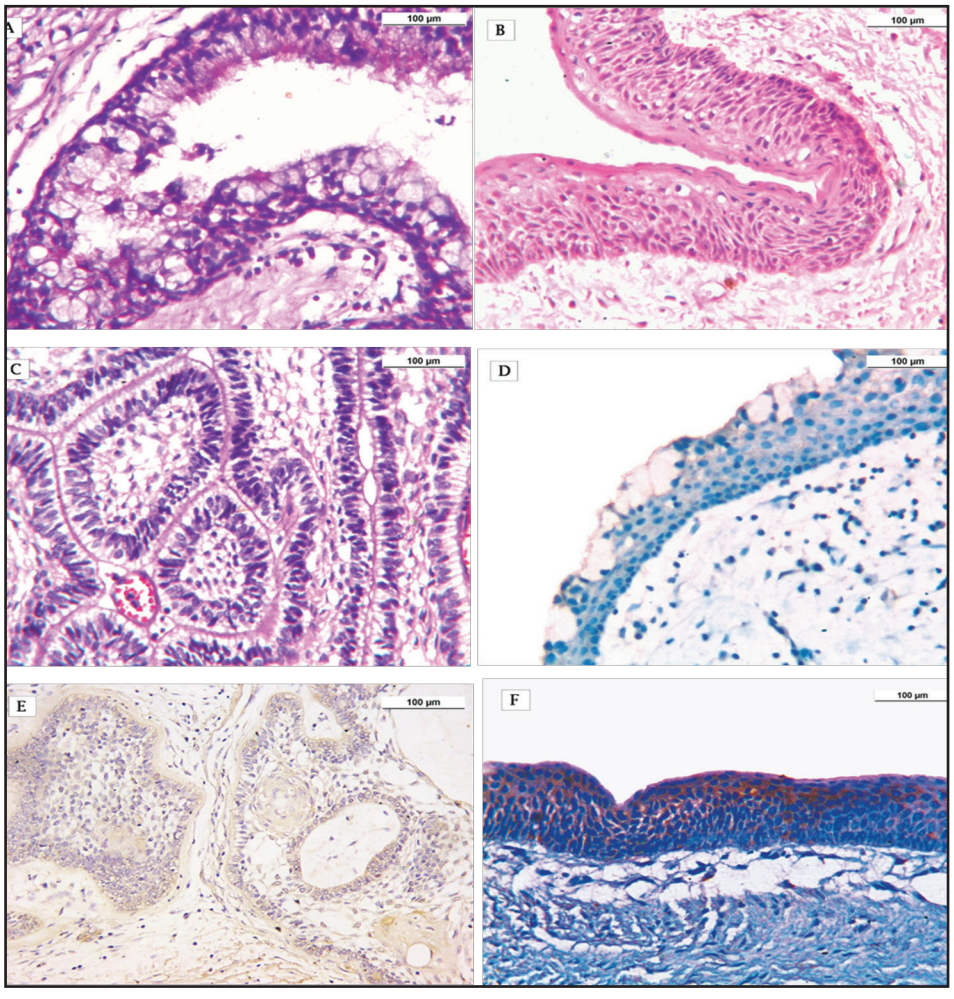

Figure (1) H&E staining of DC, AB and OKC (A, B and C respectively, X200). KLK3 immunostaining in DC, AB and OKC (D, E and F respectively, X 200)

composed of cuboidal or columnar epithelial cells with a palisaded polarized layer and nuclei with hyperchromatism **Fig. (1C)**.

Immunohistochemical finding

Slight KLK3 cytoplasmic immunostaining was seen in the epithelial cells of the cystic lining of DC also no stromal staining of KLK3 **Fig. (1D).** All cases of AB showed cytoplasmic positivity in epithelial cells. KLK3 was expressed at the basal end of ameloblast-like cells**.** Patchy Stromal positivity was also observed around the follicles. Moreover, cytoplasmic KLK3 immunoexpression was observed in stellate reticulum-like cells of AB **Fig. (1E).** OKC were positive to the KLK3 immunostaining. Most of the cases of OKC showed cytoplasmic expression of KLK3. The cytoplasmic KLK3 expression was noted among different layers of the epithelial lining **Fig. (1F).**

The greatest mean area percent was recorded in AB (49.56), then OKC (25.99) whereas the lowest mean was recorded in DC (12.90) **Fig. (2)**. An extremely statistically significant ($p<0.001$) was revealed using ANOVA test. The highest gene expression was recorded in AB (3.45) then OKC (2.22) and the lowest gene expression was seen in DC (0.85) **Fig. (3).** The best cut-off value for detection of cases using kallikrein 3 area percent was 19.135 with sensitivity=100%, specificity=100% and overall accuracy=100% **Fig. (4).** The best cut-off value for detection of cases using kallikrein 3 RT-PCR was 1.3 with sensitivity=100%, specificity=100% and overall accuracy=100% **Fig. (5) Tab. (1)**

Table (1) *Descriptive statistics of diagnostic accuracy of both techniques RT-PCR and immunostaining*.

Test Result Variable(s)	Area under curve	P value	95% Confidence Interval		Cut off value	Sensitivity	Specificity	PPV	NPV	Accuracy
			Lower Bound	Upper Bound						
kallikrein 3 area percent	1.000	<0.001	1.000	1.000	19.135	100%	100%	100%	100%	100%
kallikrein 3 RT-PCR	1.000	<0.001	1.000	1.000	1.3	100%	100%	100%	100%	100%

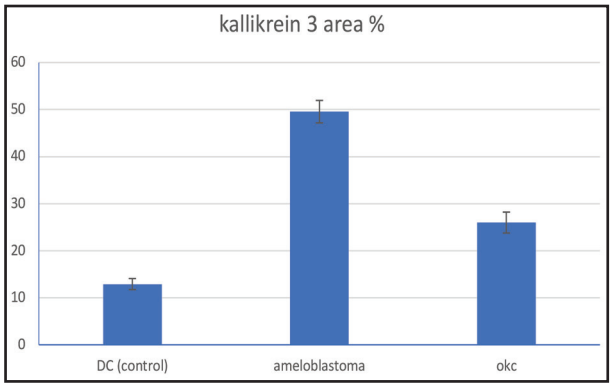

Figure (2): Bar chart illustrating mean area percent between AB, OKC and DC

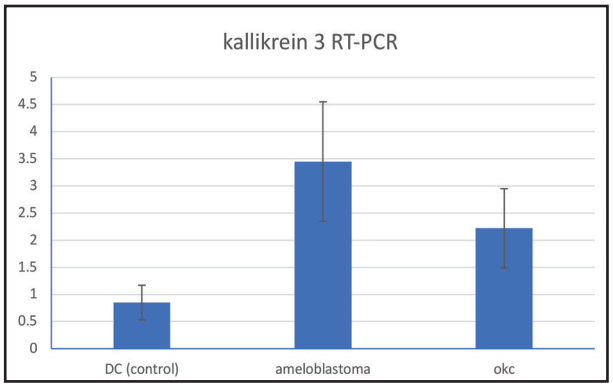

Figure (3): Column charts showing KLK3 gene expression comparing between AB, OKC and DC

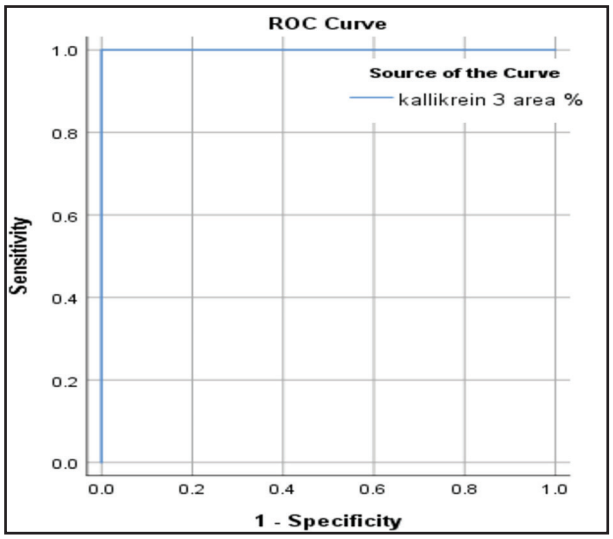

Figure (4) ROC curve for detection of cases using kallikrein 3 area percent

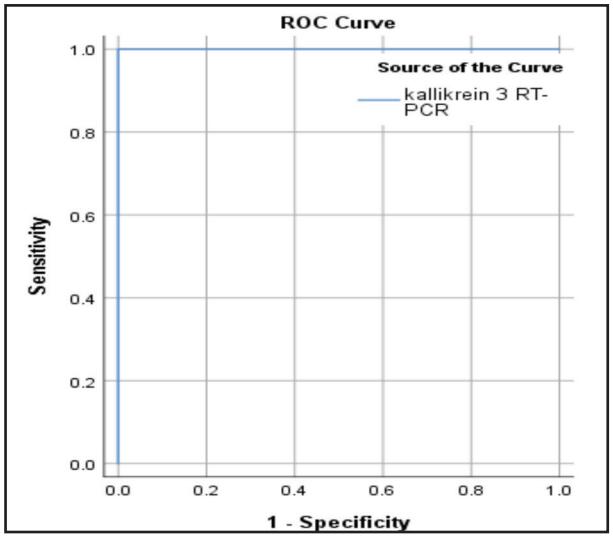

Figure (5) ROC curve for detection of cases using kallikrein 3 RT-PCR

DISCUSSION

Regarding the current study, the immunohistochemical expression of KLK3 was found (49.56 ± 2.39) in AB, (25.99 ± 2.22) in OKC and the lowest recorded was in DC (12.90 ± 1.18). Also, the gene expression of KLK3 was found (3.45± 1.10) in AB, (2.22± 0.73) and the lowest KLK3 gene expression was recorded in DC (0.85 ± 0.32). Also, the statistical analysis showed highest expression of KLK3 in AB and while the lowest expression was found in DC. There was statistically significant difference between the mean values of AB in relation to OKC and DC. The cut-off values for detection of cases using KLK3 area % was 19.135 with sensitivity=100%, specificity=100% and overall accuracy=100%, cut-off value for detection of cases using KLK3 RT-PCR was 1.3 with sensitivity=100%, specificity=100% and overall accuracy=100. Comparing the sensitivity of both techniques together revealed no statistically significant difference.

These results were similar to a study which reported that staining AB and OKC using KLK3 shows greater staining in AB, and OKC compared with controls, these results suggested that KLK 3 might have important role in their formation. In AB, a patchy staining of the connective tissue stroma was seen, this finding is reliable with the fact that KLK 3 is a secreted protein. The increased expression in AB and OKC advocates that KLK 3 may have a role in their intrinsic and uncontrollable growth rates compared to less clinically aggressive odontogenic cysts[18]. KLK3 greater expression was found in ABs ($p<0.01$) and OKCs ($p<0.05$) than in the odontogenic control. Also, KLK3 was found to show a greater expression in AB and OKC, when compared to the odontogenic hamartoma. However, the study was not consistent with the RT-PCR results. Application of RT-PCR technique showed no recognition of KLK 3 mRNA in AB, the reason may be that KLK 3 protein may be engaged or may sustain for long time in benign tumours with odontogenic origin.

KLKs are expressed in many different tissues at both levels the mRNA and protein. KLK shows a relatively wide expression outline in tissues, with high expression value within a little major tissues and low expression level in others. Remarkably, KLKs are often co-expressed within the same tissues. Discovering KLKs in biological fluids as seminal plasma, serum, and the women lactating milk, supports that they are secreted in vivo as a

protein. Furthermore, immunohistochemistry studies indicated that KLKs, including KLK3, KLK4, KLK6, KLK7, KLK9, KLK10, are confined mostly in the glandular cytoplasmic epithelium, which are responsible for their secretion[19].

Regarding the results of the current study, it was consistent with another study in which the investigation revealed a detectable difference in the intensity of staining using KLK 3 between the evaluated lesions ($p=0.004$). By making a comparison between the odontoma to OKC ($p<0.05$) and the odontoma to AB ($p<0.01$), there was a significant difference in KLK 3 expression. Nasopalatine duct cyst, lateral periodontal cyst and DC show positive KLK 3 staining, but no significant differences were seen by making a comparison between these other lesions. Benign tumours with odontogenic origin show greater amounts of KLK3 staining. Recognition of KLK3 biomarker may help early detection and handling of local aggressive tumours[20].

Regarding the cytoplasmic expression of KLK3 in epithelial cells of AB there was a stromal patchy staining. Tumor stroma interactions that are widely known as integral to cancer progression are those between tumor cells and endothelial cells, as well as adjacent smooth muscle cells. PSA releases a kinin-like molecule from seminal fluid, even though this likely includes activation of a HMWK-activating intermediate, as recombinant PSA could not directly activate HMWK. Equally, Lys-plasminogen could be cleaved by PSA to release bioactive fragments, and these purified peptides prevent human umbilical vein endothelial, HUVEC, cell tube formation. PSA affects the expression of HUVEC-derived genes number, inversely regulating genes that are integral in tube formation[21].

On the other hand, salivary gland tumours show mild KLK3 expression. This may be attributed to different upregulations of KLKs, harmonised activation seen in salivary gland tumours or unknown mechanisms. Also, the expression profile of KLK3 in patient with malignant salivary tumours, its relation to the spreading of tumour, recurrence and prognosis, is currently being examined[22]. Another study demonstrated no KLK3 expression in salivary gland tumours and normal salivary gland tissue. This result may be due to using immunohistochemical methods alone that are not sensitive to detect KLK3 in salivary gland tissue, but a salivary duct carcinoma case with metastasis to the bone and positive for KLK3 was demarcated in a patient who shows elevated serum levels of hK3[23, 24]. PSA consumption after its secretion from the salivary gland probably caused its low concentrations[25].

It was found that KLK3 has anti-angiogenic effect, because it stops formation of endothelial cell tube on a Matrigel BM research, also affects spread and proliferation of endothelial cells in a monolayer culture. VEGF-C is activated by KLK3 specifically and efficiently by splitting at the site of novel N-terminal. VEGF-C was detected in this study in sperm liquefaction and seminal plasma, and occurred simultaneously with activation of VEGF-C, in which collagen and CCBE1 are responsible for its improvement. KLK3 is the third protease found to trigger VEGF-C After ADAMTS3 and plasmin[26].

The presence of statistically significantly increased KLK 3 protein in these tumours calls for further investigations. Learning about this protein may contribute to an understanding of the more aggressive behaviour observed in AB and OKC compared to odontogenic cysts. The previous data and experimental design do not readily support any specific mechanism of action of KLK3, however potential roles could include KLK3 acting as a part of a larger cascade of molecular signalling involving other KLK proteins where it may function to permit local tissue destruction resulting in invasion of the lesion, or act in a cascade as an activator of growth or as an inhibitor of growth suppression. The relatively wide variety of physiologic processes and pathways of molecular signalling identified to be associated with KLKs suggest that KLK3 could be involved in the pathogenesis of these lesions.

CONCLUSION

Increased expression of KLK3 mRNA in AB and OKC suggests that KLK3 may have a role in the intrinsic and limitless growth potential of these lesions.

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RECOMMENDATION

The presence of statistically significantly increased KLK 3 protein and KLK3 mRNA gene in these tumors would be interesting to further investigate. Studying this gene may contribute to an understanding of the more aggressive behavior observed in the AB and OKC compared to other developmental odontogenic cysts. Further studies on larger numbers of samples are required to confirm the relation of KLK3 protein and KLK3 gene in the aggressive behavior of AB and OKC.

CONFLICT OF INTEREST

None declared

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