Western University
Scholarship@Western

Paediatrics Publications | Paediatrics Department

3-1-2021

Human Tissue Kallikreins in Polymorphous Adenocarcinoma: A Polymerase Chain Reaction and Immunohistochemical Study

Jacqueline Cox
Schulich School of Medicine & Dentistry

Zia Khan
Schulich School of Medicine & Dentistry, zia.khan@schulich.uwo.ca

Linda Jackson-Boeters
Schulich School of Medicine & Dentistry

Jerrold Armstrong
Schulich School of Medicine & Dentistry

Mark Darling
Schulich School of Medicine & Dentistry

Follow this and additional works at: https://ir.lib.uwo.ca/paedpub

Citation of this paper:
Cox, Jacqueline; Khan, Zia; Jackson-Boeters, Linda; Armstrong, Jerrold; and Darling, Mark, "Human Tissue Kallikreins in Polymorphous Adenocarcinoma: A Polymerase Chain Reaction and Immunohistochemical Study" (2021). *Paediatrics Publications*. 2016.
https://ir.lib.uwo.ca/paedpub/2016

Head and Neck Pathology (2021) 15:169–178
https://doi.org/10.1007/s12105-020-01196-2

Human Tissue Kallikreins in Polymorphous Adenocarcinoma: A Polymerase Chain Reaction and Immunohistochemical Study

Jacqueline Cox[1] · Zia Khan[1] · Linda Jackson-Boeters[1] · Jerrold Armstrong[1] · Mark Darling[1,2]

Received: 25 May 2020 / Accepted: 20 June 2020 / Published online: 26 June 2020
© Springer Science+Business Media, LLC, part of Springer Nature 2020

Abstract
Polymorphous adenocarcinoma (PAC) is the second most common malignant salivary gland tumour of minor salivary glands. Human tissue kallikreins (KLKs) are a family of highly conserved serine proteases expressed by various tissues and organs. The literature demonstrates a link between KLKs and salivary gland neoplasms. The purpose of this study was to determine levels of KLK mRNA in tissue samples of PAC and to determine if KLK expression is limited to tumour cells. Nineteen cases of PAC were reviewed (1987–2013). The diagnosis was confirmed, demographic data was collected, and formalin fixed paraffin-embedded PAC and normal salivary gland tissue samples were obtained. RNA isolation was achieved, followed by conversion to complementary DNA via reverse transcription. Using PCR, the quantitative level of expression of KLKs1–15 was recorded. Samples exhibiting high and low KLK expression were selected for immunohistochemistry staining. Results revealed a statistically significant increase in mean KLK mRNA expression for KLK1, KLK4, KLK10, KLK12 and KLK15 in PAC tissue samples, compared with normal salivary gland tissue (Mann–Whitney U test, $p < 0.05$). Immunohistochemistry results demonstrated that KLKs were present in tumor cells. Notably, all samples demonstrating relatively higher KLK mRNA expression showed equivalent or increased staining scores relative to the low KLK mRNA expression samples. In conclusion, there appears to be aberrant kallikrein expression in polymorphous adenocarcinoma, suggesting the possibility of a kallikrein cascade influence on tumor development and progression.

Keywords Tissue kallikreins · Immunohistochemistry · Salivary gland neoplasms · Adenocarcinoma · Reverse transcriptase polymerase chain reaction

Introduction

Salivary gland tumours comprise 2–6.5% of all head and neck neoplasms and have a global incidence of approximately 1–6.5 cases per 100,000 persons annually [1]. Polymorphous adenocarcinoma (PAC) is a malignant epithelial tumour characterized by cellular uniformity, morphologic diversity, an infiltrative growth pattern, and low metastatic potential [2]. It is noteworthy that in the latest World Health Organization (WHO) classification, the term "low-grade" was dropped from its previous designation of "polymorphous low-grade adenocarcinoma", to allow for flexibility in grading [2]. Most commonly, it presents as a firm, non-tender swelling of the palate, buccal or lip mucosa [3].

The diagnostic challenge PAC poses is its histomorphologic resemblance and growth pattern overlap with pleomorphic adenoma (PA) and adenoid cystic carcinoma (ACC) [4–6]. In an attempt to improve diagnostic accuracy in salivary gland pathology, many groups have examined the immunohistochemical profile of PAC and its associated biomarkers; however, none of these biomarkers have been used independently in the diagnosis and prognosis of PAC [7]. PRKD1 is a gene that encodes a kinase protein, which has been implicated in conferring a neoplastic advantage to PAC tumour cells [6].

Human kallikreins (KLKs) are secreted serine proteases, with a range of physiologic and pathologic functions [8]. They are 15 of 178 human serine proteases belonging to the S1A subfamily [9]. KLKs have emerged as powerful tumour

✉ Mark Darling
mdarlin@uwo.ca

[1] Schulich School of Medicine and Dentistry, University of Western Ontario, London, ON, Canada

[2] Department of Pathology and Laboratory Medicine, University of Western Ontario, 1151 Richmond Street, HSA 424, London, ON N6A 5C1, Canada

markers, with the most well-known biomarker being KLK3 (prostate specific antigen or PSA), a valuable aid for prostate cancer diagnosis and monitoring [8–11]. KLKs have been studied as potential biomarkers for a variety of other malignancies, and specific KLKs have demonstrated association with an unfavourable prognosis. KLKs4–7, KLK10, KLK11, and KLK15 have been used as biomarkers for ovarian cancer; KLK5, KLK7, KLK10 and KLK14 for breast cancer; and KLK7, KLK10, and KLK14 for colon cancer [9, 12–18]. To date, no KLK has been used as a biomarker in salivary gland malignancies though most have been detected in salivary gland tissue. KLKs are potentially useful biomarkers to aid in diagnosis, prognosis, and monitoring of salivary gland neoplasms [19–22].

We hypothesize that specific KLKs are aberrantly regulated in PAC, as compared to normal salivary gland tissue (NSGT). Furthermore, we postulate that KLK expression, whether increased or decreased, may be demonstrated specifically in tumour cells.

This study aimed to determine levels of KLK (KLKs1–15) mRNA in formalin fixed paraffin embedded PAC tissue cases, using real time polymerase chain reaction. A second aim was to determine if KLK expression is tumour cell specific, by analyzing immunohistochemical expression of specific KLKs in PAC tissue cases.

The study was approved by the Western University Research and Ethics Board, Project ID 103783. The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki.

Materials and Methods

Tumour Criteria

Cases of polymorphous adenocarcinoma (PAC) were retrieved from the archives between 1987 and 2013 at University Hospital, London Health Sciences Centre and the Division of Oral Pathology, Schulich School of Medicine and Dentistry, Western University. Original hematoxylin and eosin (H&E) stained slides were reviewed to confirm a diagnosis of PAC. Blocks of formalin fixed, paraffin embedded (FFPE) PAC tissues were obtained for suitable cases. A total of 19 PAC tumors were obtained.

Patient Data Collection from PAC Cases

Data gathered included patient age, gender, tumour clinical features (site, recurrence) and microscopic findings (encapsulation, degree of infiltration, morphology, perineural invasion). Patient demographics are summarized in Table 1.

Table 1 Patient demographics

	All specimens			
n	19			
Age range	17–83			
Gender	Female		Male	
	13		6	
Tumour location	Palate	Buccal mucosa	Lip	Sublingual gland
	11	5	2	1

RNA Isolation from Formalin-Fixed, Paraffin-Embedded Tissue

Regions representative of PAC tissue were circled and matched to the same region on the FFPE tissue block. A microtome (Microm HM 325, GMI, Inc., Ramsey, MN, USA, SKU# 8243 30 1006) was used to prepare 3 × 10 µm tissue curls from each of the PAC FFPE tissue blocks and were then placed in 1.5 mL collection tubes. KLK mRNA expression in normal salivary gland tissues was used as a control. Tissues were deparaffinised and subsequent centrifugation was completed for 2 min at maximum speed (12,000–14,000 × g). The supernatant was discarded and centrifugation was repeated with subsequent removal of residual fluid. Cases were then incubated with the collection tube open in a heating block for 5–15 min at 55 °C to air dry the tissue pellet.

High pure FFPE RNA Micro Kit (Roche Applied Science, Mannheim, Germany, Catalogue Number 04 823 125 001) was used to isolate RNA from each of the cases, following manufacturer's instructions. Pure total RNA was obtained as a result and was stored at − 80 °C.

Isolated pure total RNA was centrifuged for 2 min at maximum speed; the supernatant was transferred to a fresh collection tube to be quantified. Qubit Fluorometer (Invitrogen, Carlsbad, CA, Catalogue No. Q32866) was used to measure RNA. A minimum of 0.32 ng of RNA was required for further experimentation.

cDNA Synthesis from Isolated RNA

The iScript cDNA synthesis kit [Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON, Catalogue Number 1708890] was used to synthesize complementary DNA (cDNA) from previously isolated RNA. A master tube of iScript Reaction Mix and iScript Reverse Transcriptase was prepared. Reaction tubes were prepared, each with a variable amount of tumour RNA and cDNA

Table 2 cDNA synthesis

Case number	Enzyme/ buffer mix (μL)	RNA (μL)	Nuclease-free H_2O (μL)	Total volume (μL)
1	5	3.5	11.5	20
2	5	2.5	12.5	20
3	5	4.0	11.0	20
4	5	4.0	11.0	20
5	5	1.5	13.5	20
6	5	0.5	14.5	20
7	5	2.5	12.5	20
8	5	5.0	10.0	20
9	5	1.0	14.0	20
10	5	1.5	13.5	20
11	5	0.5	14.5	20
12	5	1.5	13.5	20
13	5	0.5	14.5	20
14	–	Too low	–	–
15	5	3.0	12.0	20
16	5	1.0	14.0	20
17	5	2.0	13.0	20
18	–	Too low	–	–
19	–	Too low	–	–

was synthesized according to manufacturer's instructions (Table 2).

SYBR Green Real Time Polymerase Chain Reaction (PCR)

The CFX Connect Real Time PCR Detection System [Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON] was used to run the RT-qPCR reactions. Qiagen provided custom 96 well PCR plates, each containing primer assays for KLK1–15 and β-actin (reference gene) (Table 3). Six PAC cases were added per plate. RT2 SYBR® Green qPCR Mastermix (Qiagen, Toronto, ON, Catalogue No. 330500) was used to complete the real-time PCR experiment and contained the following reagents: RT-PCR buffer, hot start Taq DNA polymerase, nucleotides, and SYBR green dye. Data generated was analyzed by ΔΔCT using CFX Manager. For each KLK, the mean of mRNA expression in tumour cases was calculated, as was the standard error of mean. Non-parametric statistical analysis, specifically Mann–Whitney U Test, was completed. Statistical significance was set at $p < 0.05$.

Table 3 Qiagen catalogue numbers for primer assays used in custom PCR plate

Primer assay	Catalogue number
KLK1	QT00020664
KLK2	QT00088466
KLK3	QT00027713
KLK4	QT00495159
KLK5	QT00010437
KLK6	QT00013972
KLK7	QT00028343
KLK8	QT00017689
KLK9	QT00057190
KLK10	QT00039816
KLK11	QT00011011
KLK12	QT00067977
KLK13	QT00029876
KLK14	QT00039928
KLK15	QT00035735
β-Actin	QT01680476

Immunohistochemistry

Case Selection

Cases were selected for immunohistochemistry after analyzing KLK mRNA levels from PCR data and tissues exhibiting relatively lower or higher mRNA levels of specific KLKs were included. KLK antibodies, including rabbit polyclonal antibodies were obtained from the Diamandis Lab (University of Toronto, ON, Canada). Table 4 outlines the cases used for immunohistochemistry experiments.

Paraffin Sections

Formalin fixed, paraffin embedded PAC tissue blocks from cases of interest were obtained for immunohistochemistry. 5 μm Thick serial sections were cut using a microtome (Microm HM 325, GMI, Inc., Ramsey, MN, USA, SKU# 8243 30 1006).

Table 4 Samples used for immunohistochemistry experiments

Specific KLK antibody	Case # Low level	Case # High level
KLK7	6	16
KLK8	6, 16	11
KLK10	11	6, 10
KLK11	6	11
KLK14	6	11

Staining Procedure

A standard immunohistochemical staining method was employed. An indirect technique was used to localize tissue expression of cellular antigens, specifically expression of KLK7, KLK8, KLK10, KLK11, and KLK14 in PAC tissue cases of interest. Positive controls were used for each KLK antibody to ensure sensitivity, and included the following: ACC for KLK7, skin for KLK8 and KLK14, NSGT for KLK10, and prostate for KLK11. Negative controls of the PAC tissue were obtained by omitting the primary antibodies from the experiments. Optimal dilutions were as follows: 1:3200 for KLK7 and KLK14, 1:100 for KLK8, 1:1200 for KLK10, and 1:2400 for KLK11.

Tissue sections were subjected to antigen retrieval, utilizing citrate buffer (pH 6.00), at 125 °C in a de-cloaking chamber. The slides were rinsed in water, then PBS for 5 min and subsequently blocked, using 10% horse serum at room temperature. After 30 min, the appropriately diluted primary antibodies, for the KLK of interest, were added to the slides and incubated for the outlined optimal time. Excess antibody was removed with a PBS rinse for 5 min. The ImmPRESS kit (ImmPRESS ® Reagent Kit; Vector Laboratories, Burlingame, CA, Catalogue No. MP-7401) provided the anti-rabbit antibodies, conjugated with horseradish peroxidase micropolymers; tissue sections were incubated with the anti-rabbit antibodies for 30 min at room temperature. The slides were then stained with 3, 3′-diaminobenzidine tetrahydrochloride (DAB Peroxidase Substrate Kit, Vector Laboratories, Burlingame, CA, Catalogue No. SK-4100) as the chromogen, followed by a hematoxylin counterstain.

Data Collection and Statistical Analysis for Immunohistochemistry

The KLK staining of each PAC case was viewed under light microscopy and assessed semi-quantitatively, using a well described method [21]. A proportion score (0–5), and an intensity score (0–3) was used to evaluate each stained tissue case. These scores were then used to generate an overall staining score (OSS).

Results

RNA Isolation

The RNA isolated from PAC cases is shown in Table 5. In cases 14, 18, and 19, the RNA amount was not detectable. The average amount of RNA obtained from the remaining 16 cases was 0.297 µg/mL.

Table 5 RNA quantification by Qubit Fluorometer for each PLGA tissue sample

Case number	RNA (µg/mL)
1	0.091
2	0.121
3	0.075
4	0.072
5	0.214
6	0.898
7	0.139
8	0.065
9	0.322
10	0.244
11	1.04
12	0.238
13	0.520
14	Too low
15	0.120
16	0.426
17	0.163
18	Too low
19	Too low

Table 6 Percentages of PLGA tissue samples expressing KLK1–15 and calculated p-values, as determined by Mann–Whitney U Test, for the comparison of expression of KLK1–15 in PLGA tissues and NSGT

KLK	PLGA (%)	p-value
KLK1	87.5	0.00672
KLK2	87.5	0.82588
KLK3	62.5	0.59612
KLK4	43.8	0.03236
KLK5	68.8	0.238
KLK6	25	Sample number low
KLK7	18.8	Sample number low
KLK8	31.3	0.15854
KLK9	50	0.71138
KLK10	62.5	0.00804
KLK11	25	Sample number low
KLK12	68.8	0.0536
KLK13	25	Sample number low
KLK14	37.5	0.88076
KLK15	37.5	0.00386

Expression of KLKs in cDNA of PAC

The expression of KLK mRNA in cases of PAC was examined for KLK1–15. All cases of PAC tissue demonstrated expression of at least 4 KLKs, with some showing as many as 13 KLKs. The percentages of PAC tissues exhibiting each KLK are listed in Table 6. KLK7 was the least likely to be expressed in the cases, having just 18.8% expression. KLK6,

KLK11, and KLK13, having 25% expression in tissue cases, were also expressed infrequently. KLK1 and KLK2 were most commonly expressed.

Expression of KLKs in PAC Compared to Normal Salivary Gland Tissue (NSGT)

The mean expression of each KLK was calculated for all PAC cases. Figure 1a–e demonstrates statistically significant KLK mRNA expression in PAC tissues, as compared to NSGT. Statistical significance was determined using Mann–Whitney U Test, $p < 0.05$. For each KLK, the mean mRNA expression was found to be greater in the PAC cases, compared with the NSGT. This was statistically significant for KLK1, KLK4, KLK10, KLK12, KLK15. The relative fold change of mRNA expression for each KLK in PAC versus NSGT is demonstrated in Fig. 1f. In each graph, the bar-lines indicate the standard error of mean (SEM).

Immunohistochemical Staining Results

Representative immunohistochemical staining images are shown in Fig. 2 of PAC tissue cases stained for particular KLKs. For each KLK antibody, at least one PAC case demonstrating relatively high mRNA expression is presented. For each KLK antibody, at least one PAC case demonstrating relatively low mRNA expression and one PAC case demonstrating relatively high mRNA expression are presented.

The IHC staining results are outlined in Table 7, with total IHC scores for each case demonstrated in Fig. 3a. Cases demonstrating relatively high KLK expression exhibited staining of all tumour cells (grade 5) and the intensity grade of the stains varied from 1 to 3. Cases demonstrating relatively low KLK expression exhibited staining of most tumour cells (grades 4–5) and the intensity grade of the stains varied from 1 to 3. With respect to localization of a specific KLK, the cases with higher mRNA expression demonstrated equivalent or increased immunohistochemical staining, as compared with cases with lower mRNA expression. Most of the IHC stains on PAC cases showed primarily cytoplasmic staining, with some exceptions. Nuclear cell staining was seen with KLK7 (cases 6 and 16), KLK11 (cases 6 and 11), and KLK14 (cases 6 and 11). KLK10 exhibited inflammatory cell staining in case 10, and epithelial cell staining in case 11 (Fig. 3b). All cases demonstrated negative stromal staining. Results from PCR KLK mRNA quantification and IHC staining were compared and the findings are summarized in Table 8.

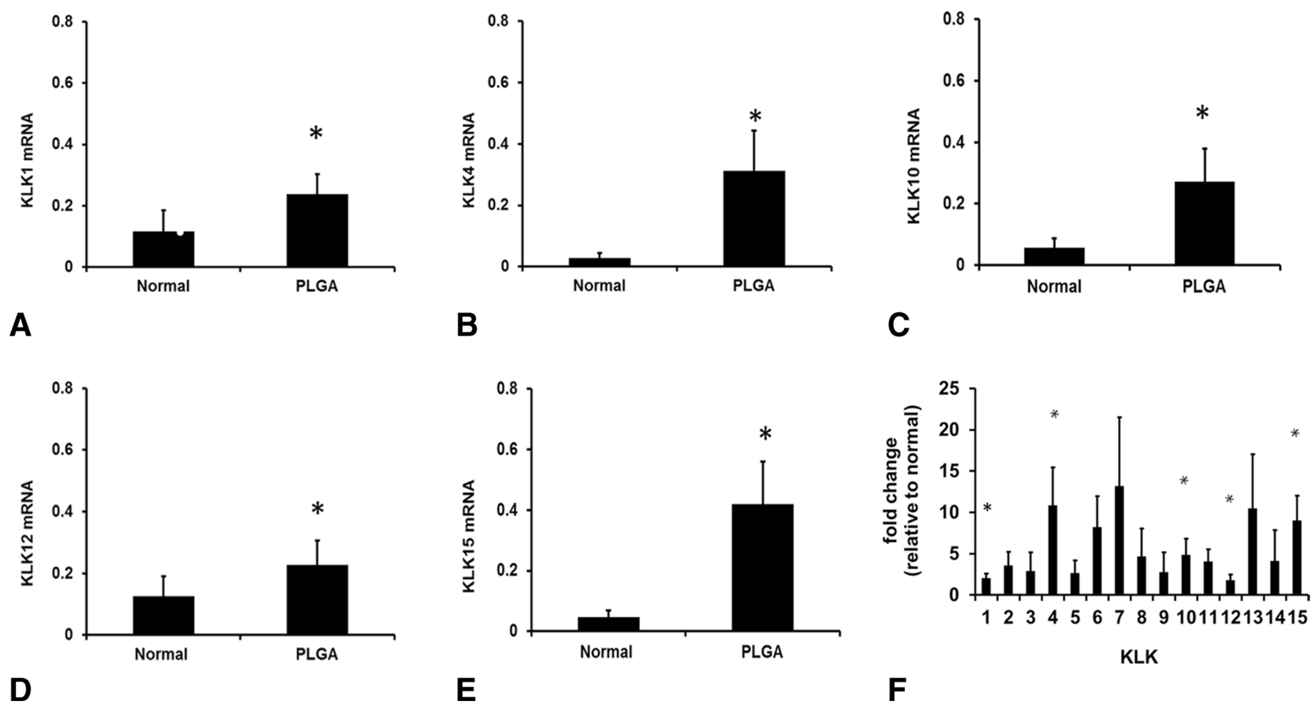

Fig. 1 **a–e** Mean value of KLKs1, 4, 10, 12, and 15 mRNA expression in PLGA vs NSGT and **f** relative fold increase of mRNA expression of KLK1-15 in PLGA tissue as compared with NSGT. Statistical significance determined by Mann–Whitney U Test ($p < 0.05$), standard error of mean (SEM) is demarcated by the graph bars

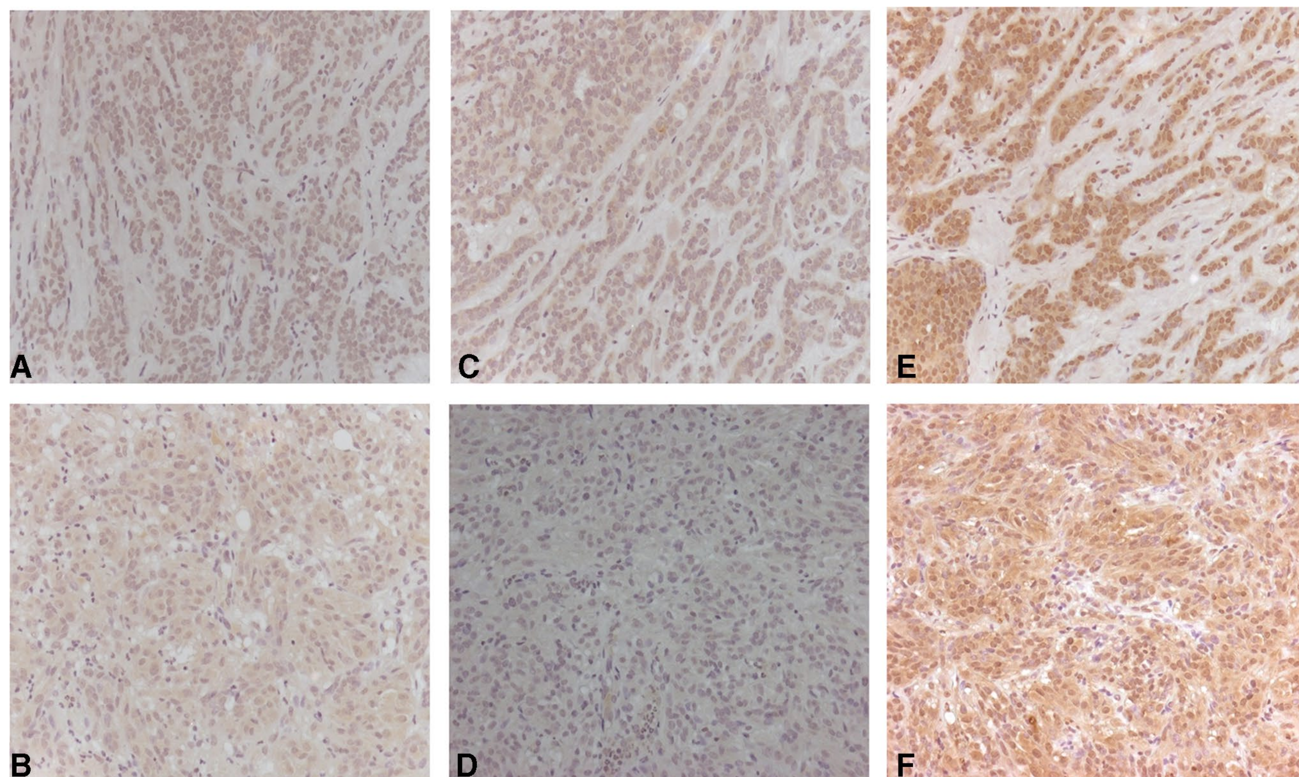

Fig. 2 (Left) comparative immunohistochemical localization of KLK8 in PLGA, cases 6 and 11. **a** Case 6 demonstrated relatively low KLK8 mRNA expression on PCR, **b** case 11 demonstrated relatively high KLK8 mRNA expression on PCR. (Centre) comparative immunohistochemical localization of KLK10 in PLGA, cases 6 and 11: **c** case 6 demonstrated relatively high KLK10 mRNA expression on PCR, **d** case 11 demonstrated relatively low KLK10 mRNA expression on PCR. (Right) comparative immunohistochemical localization of KLK14 in PLGA, cases 6 and 11: **e** case 6 demonstrated relatively low KLK14 mRNA expression on PCR, **f** case 11 demonstrated relatively high KLK14 mRNA expression on PCR (images taken at × 200 original magnification, blue hematoxylin nuclear stain, brown diaminobenzidine/DAB chromogen)

Table 7 Immunohistochemistry (IHC) proportion score, intensity and overall score are recorded below, along with specific staining characteristics seen in each sample

Antibody	Case #	Level of mRNA	Proportion score	Intensity score	IHC total score	Stromal stain	Other
KLK7	6	Low	5	2	7	–	Cytoplasmic and nuclear staining
KLK7	16	High	5	2	7	–	Cytoplasmic and nuclear staining
KLK8	6	Low	4	1	5	–	Primarily cytoplasmic staining
KLK8	16	Low	4	1	5	–	Primarily cytoplasmic staining
KLK8	11	High	5	1+	6+	–	All cytoplasmic staining
KLK10	11	Low	5	1/2	5.5	–	Epithelial positive cells
KLK10	6	High	5	1	6	–	All cytoplasmic staining
KLK10	10	High	5	1	6	–	All cytoplasmic staining; inflammatory cells staining positively
KLK11	6	Low	5	2	7	–	Primary cytoplasmic staining; many nuclei staining
KLK11	11	High	5	3	8	–	Primary cytoplasmic staining; some positive and negative nuclei
KLK14	6	Low	5	3	8	–	Primarily cytoplasmic staining; some nuclei staining
KLK14	11	High	5	3	8	–	Primarily cytoplasmic staining; much nuclear staining

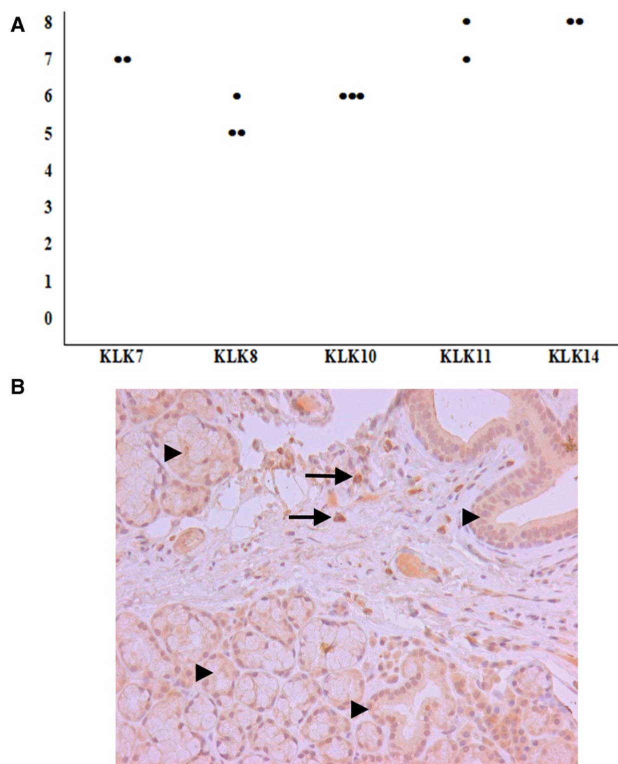

Fig. 3 **a** Immunohistochemistry stain results. IHC total score seen in each case is shown. **b** Kallikrein 10 staining in salivary gland. Faint staining is present in ducts and acinar cells (arrowheads), and some staining is present in inflammatory cells (arrows) (×200 original magnification, blue hematoxylin nuclear stain, brown diaminobenzidine/DAB chromogen)

Discussion

PCR Data Suggests an Increase in all KLK Expression in PAC

Through PCR we have demonstrated that KLK expression is upregulated in PAC. The average levels of KLK1–15 mRNA expressed in all PAC tissue cases were increased relative to NSGT. This upregulation was statistically significant for KLK1, KLK4, KLK10, KLK12, and KLK15 ($p < 0.05$). KLK1 had the most statistically significant upregulation in PAC as compared to NSGT with a p-value of 0.00672. In particular, KLK6, KLK7, KLK11 and KLK13 exhibit wide error bars; this is indicated by the lower percentages of cases expressing these KLKs: 25%, 18.8%, 25% and 25%, respectively.

IHC Demonstrates that KLK Upregulation is Specific to PAC Tumour Cells

Immunohistochemistry experiments were used in the current study to determine if KLK upregulation in PAC was

Table 8 Summary of PCR and IHC results for selected cases

	Total PLGA samples		Case 6		Case 10		Case 11		Case 16	
	Average level of mRNA	Median value of mRNA	mRNA	IHC score	mRNA	IHC score	mRNA	IHC score	mRNA	IHC score
KLK7	0.45908	0.37725	Low, 0.0	7					High, 0.377251	7
KLK8	0.25982	0.09694	Low, 0.0969368	5			High, 1.0	7	Low, 0.1049704	5
KLK10	0.27191	0.12312	High, 0.225234	6	High, 0.138793	6	Low, 0.048942	6		
KLK11	0.51035	0.44622	Low, 0.0	7			High, 1.0	8		
KLK14	0.18320	0.02862	Low, 0.02862	8			High, 1.0	8		

tumour cell-specific. Proportion scores for each PAC case stained with KLK antibody revealed that most tumour cells were staining positively with a proportion score 5, save for two cases with KLK8 demonstrating scores of 4, indicating a slightly lower percentage of tumour cells taking up stain. Intensity scores varied between cases. Ultimately, the experiment was able to demonstrate that the upregulation of KLKs in PAC was tumour-cell specific.

KLK mRNA Profile of PAC vs ACC, as Determined by PCR

Recently, the expression of KLKs in ACC and PA has been examined [23, 24]. Kerr et al. concluded that KLK1, KLK8, KLK11 and KLK14 were significantly downregulated in ACC, as compared to NSGT [23]. In the present study, KLK1, KLK4, KLK10, KLK12, and KLK15 were found to be significantly upregulated in PAC, when compared to NSGT. These differences, specifically KLK1, which was present in 73% of ACC cases studied by Kerr et al., and in the present study, expressed in 87.5% of PAC cases, may prove to be useful in distinguishing PAC from ACC [3–5, 23].

Morrison et al. recently investigated the KLK expression profile in PA and found that mRNA expression of KLK1, KLK12 and KLK13 was downregulated relative to NSGT ($p < 0.05$) [24]. This finding was supported in immunostaining studies. Interestingly, a relative increased KLK1 protein expression was noted in PA cases demonstrating capsular perforation [24]. Taken together with the findings of the present study, which noted an upregulation in KLK1 in PAC, KLK1 may infer more aggressive tumour behaviour. These differences in IHC staining profiles may be useful in diagnostic distinction between PAC and PA.

Analysis of IHC Staining of KLKs in PAC

Overall, each case with a relatively higher expression of KLK mRNA stained equivalently or with a higher IHC score than the cases which had relatively lower KLK mRNA expression. For KLK7, case 6 demonstrated no mRNA expression, and case 16 showed relatively higher expression; however, both cases had equivalent IHC scores of 7. Similarly, for KLK10, case 11 had relatively low mRNA expression in comparison to cases 6 and 10, yet the IHC score for all cases was 6. Furthermore, for KLK14, case 6 had relatively low mRNA expression in comparison to case 11; IHC scores for both cases were 8. A small increase was seen between cases for KLK11, with case 6 demonstrating zero mRNA and an IHC score of 7, while case 11 having relatively higher mRNA expression and an IHC score of 8. The most significant difference was seen with KLK8; cases 6 and 16 demonstrated relatively lower mRNA levels and had IHC scores of 5, while case 11 had relatively high mRNA and an IHC score of 7. Additional IHC staining results note that all cases demonstrated negative stromal staining.

The no to minimal differences in IHC scores between cases with low and high mRNA expression may be explained by a number of postulations. Reverse transcriptase PCR determines the amount of mRNA, which is indicative of transcription of KLK genes; however, IHC involves localizing the KLK protein in the tumour cells. It is possible that while the tumour cells are increasing the rate of transcribing the KLK genes, it can lead to prohibiting translation and as a result, no protein is being made. Furthermore, we know KLKs are secreted proteases, and the process of secretion may be carried out at different rates, affecting the IHC staining characteristics.

IHC Staining of KLKs in Salivary Gland Neoplasia

KLK IHC staining profiles of salivary gland neoplasia, as well as NSGT, have been well documented, including KLK7, KLK8, KLK10 and KLK14. Hashem et al. noted that KLK7 was significantly overexpressed in PA and ACC (OSS 7.0 and 7.5 respectively), when compared to NSGT (OSS 6.5) [25]. The PAC cases in this study, whether they had relatively low or higher mRNA expression, both had OSS of 7.0, which we can extrapolate as significantly increased as compared to NSGT. In previous work by Darling et al., KLK8 was shown to be expressed in NSGT (OSS 7.0), and to demonstrate equal or increased OSS in most malignancies: ACC (OSS 7.0 +/− 1.4), PAC (OSS 6.9 +/− 0.5), ACI (OSS 7.0 +/− 0.8), ANOS (OSS 7.6 +/− 0.7) [20]. In the present study, OSS in relative low-expressing KLK8 mRNA cases was 5, and in relatively higher expressing KLK8 mRNA cases was 7, which is reasonably consistent with previous work. Darling et al. also described KLK10 as found in NSGT (OSS 6.0), and no significant differences in KLK10 expression were seen in malignancy: ACC (OSS 5.8) [21]. PAC was not investigated in Darling et al.'s study; however, in the present study, the OSS for KLK10 of our PAC cases were consistent with the other salivary gland malignancies (OSS 6 for all three cases). Hashem et al. noted KLK14 was present in NSGT (OSS 6.5), and that, while KLK14 was upregulated in PA (OSS 7.25), there were no significant differences seen in salivary gland malignancies: ACC (OSS 6.8) [22]. KLK14 stains in our PAC cases demonstrated OSS of 8, despite differences in mRNA levels. Taken together, KLK14 may be recognized as over-expressed in PAC. Darling et al. noted KLK7, KLK10 and KLK14 appeared to be correlated in their expression in ACC, all demonstrating no significant difference from NSGT. This same correlation does not seem to be seen in our staining of PAC; KLK7 and KLK14 show similar IHC profiles, but KLK10 shows lower staining scores.

In our experiments, we did not compare NSGT and PAC staining, as we were interested in correlated data from PCR (mRNA expression levels) with IHC, look to elucidate tumour specific staining. However, by review previous works that have used the same IHC protocol and staining scoring systems, this present study has added value to further characterizing KLK IHC staining profiles in salivary gland neoplasia.

Conclusion

Our study confirms the hypothesis that PAC demonstrate aberrant expression of KLKs. We believe KLK upregulation in PAC indicates the involvement of a complex proteolytic cascade, and may more significantly involve KLK1, KLK4, KLK10, KLK12, and KLK15. We suspect this upregulation of KLK activity may be involved in regulating malignant processes in PAC, and thus implicated in determining its clinical characteristics, including indolent behaviour and infrequent metastatic spread. Furthermore, the results of this study significantly add to the KLK profile of salivary gland neoplasia, at the mRNA and protein level. Importantly, when combining the resultant KLK profile in PAC with the recently elucidated KLK profile in ACC and PA, the resultant differences may render a diagnostic panel useful in differentiating between the malignancies.

Future studies should aim to identify an active proteolytic cascade involving KLK1–15 in PAC tumours. Furthermore, examining the PAC cases from our study for the PRDK1 hotspot mutation would be of value. To better elucidate the function of KLKs in PAC, it would be beneficial to study the effect of their activity or lack thereof on cancer cells.

Funding Dr. M. Darling was supported by Internal Research Grant, University of Western Ontario.

Compliance with Ethical Standards

Conflict of interest The authors have no conflict of interest to declare.

Ethical Approval Research involved formalin fixed paraffin embedded tissues only. This study was approved by the Western University Research and Ethics Board, Project ID 103783. The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki.

References

1. Neville BW, Damm DD, Allen CM, Chi AC. Oral and maxillofacial pathology. 4th ed. St. Louis: Elsevier; 2016. p. 422–72.
2. Fonseca I, Assaad A, Katabi N, Seethala R, Weinreb I, Wenig BM. Polymorphous adenocarcinoma. In: El-Naggar AK, Chan JKC, Grandis JR, Takashi T, Slootweg PJ, editors. WHO classification of head and neck tumours. 4th ed. Lyon: International Agency for Research on Cancer; 2017. p. 167–8.
3. Chi AC, Neville BW. Surface papillary epithelial hyperplasia (rough mucosa) is a helpful clue for identification of polymorphous low-grade adenocarcinoma. Head Neck Pathol. 2015;9:244–52.
4. Rooper L, Sharma R, Bishop JA. Polymorphous low grade adenocarcinoma has a consistent p63+/p40− immunophenotype that helps distinguish it from adenoid cystic carcinoma and cellular pleomorphic adenoma. Head Neck Pathol. 2015;9:79–84.
5. Persson F, Fehr A, Sundelin K, Schulte B, Loning T, Stenman G. Studies of genomic imbalances and the MYB-NFIB gene fusion in polymorphous low-grade adenocarcinoma of the head and neck. Int J Oncol. 2012;40:80–4.
6. Weinreb I, Zhang L, Tirunagari LMS, Sung YS, Chen CL, Perez-Ordonez B, et al. Novel PRKD gene rearrangements and variant fusions in cribriform adenocarcinoma of salivary gland origin. Genes Chromosome Cancer. 2014;53:845–56.
7. Strimbu K, Tavel JA. What are biomarkers? Curr Opin HIV AIDS. 2010;5(6):463–6.
8. Kontos CK, Scorilas A. Kallikrein-related peptidases (KLKs): a gene family novel cancer biomarkers. Clin Chem Lab Med. 2012;50:1877–91.
9. Obiezu CV, Diamandis EP. Human tissue kallikrein gene family: applications in cancer. Cancer Lett. 2005;224(1):1–22.
10. Pavlopoulou A, Pampalakis G, Michalopoulos I, Sotiropoulou G. Evolutionary history of tissue kallikreins. PLoS ONE. 2010;5(11):e13781.
11. Sotiropoulou G, Pampalakis G, Diamandis EP. Functional roles of human Kallikrein-related peptidases. J Biol Chem. 2009;284(48):32989–94.
12. Yousef GM, Scorilas A, Katsaros D, Fracchioli S, Iskander L, Borgono C, et al. Prognostic value of the human kallikrein gene 15 expression in ovarian cancer. J Clin Oncol. 2003;21:3119–266.
13. Borgoño CA, Grass L, Soosaipillai A, Yousef GM, Petraki CD, Howarth DHC, et al. Human kallikrein 14: a new potential biomarker for ovarian and breast cancer. Cancer Res. 2003;63:9032–41.
14. Yousef GM, Polymeris M-E, Grass L, Soosaipillai A, Chan P-C, Scorilas A, et al. Human kallikrein 5: a potential novel serum biomarker for breast and ovarian cancer. Cancer Res. 2003;63:3958–65.
15. Yousef GM, Diamandis EP. Kallikreins, steroid hormones and ovarian cancer: is there a link? Minerva Endocrinol. 2002;27:157–66.
16. Emami N, Diamandis EP. Utility of kallikrein-related peptidases (KLKs) as cancer biomarkers. Clin Chem. 2008;54:1600–7.
17. Yousef GM, Diamandis EP. Expanded human tissue kallikrein family—a novel panel of cancer biomarkers. Tumour Biol. 2002;23:185–92.
18. Emami N, Diamandis EP. Potential role of multiple members of the kallikrein-related peptidase family of serine proteases in activating latent TGF beta 1 in semen. Biol Chem. 2010;391:85–95.
19. Darling MR, Jackson-Boeters L, Daley TD, Diamandis EP. Human kallikrein 13 expression in salivary gland tumors. Int J Biol Markers. 2006;21:106–10.
20. Darling MR, Tsai S, Jackson-Boeters L, Daley TD, Diamandis EP. Human kallikrein 8 expression in salivary gland tumors. Head Neck Pathol. 2008;2(3):169–74.
21. Darling MR, Hashem NN, Zhang I, Mohamed ABD, Fung K, Kwan K, et al. Kallikrein-related peptidase 10 expression in salivary gland tissues and tumours. Int J Biol Markers. 2012;27:e381–e388388.

22. Hashem NN, Mara TW, Mohamed M, Zhang I, Fung K, Kwan KF, et al. Human kallikrein 14 (KLK14) expression in salivary gland tumors. Int J Biol Markers. 2010;25:32–7.
23. Kerr Z, Hayter A, Khan Z, Darling M. Kallikrein-related peptidase mRNA expression in adenoid cystic carcinoma of salivary glands: a polymerase chain reaction study. Head Neck Pathol. 2019. https://link.springer.com/10.1007/s12105-019-01076-4 (Epub ahead of print).
24. Morrison MD, Jackson-Boeters L, Khan ZA, Shimizu MS, Franklin JH, Fung K, et al. Identifying candidate biomarkers for pleomorphic adenoma: a case–control study. Head Neck Pathol. 2019;13:286–97.
25. Hashem N, Mohamed M, Zhang I, Fung K, Kwan K, Mara T, et al. Kallikrein-related peptidase 7 in salivary gland tumours: immunohistochemical profile. Can J Pathol. 2011;3:12–9.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.