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Kallikrein-Related Peptidase mRNA Expression in Adenoid Cystic Carcinoma of Salivary Glands: A Polymerase Chain Reaction Study

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ORIGINAL PAPER

Kallikrein-Related Peptidase mRNA Expression in Adenoid Cystic Carcinoma of Salivary Glands: A Polymerase Chain Reaction Study

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Abstract

Kallikrein-related peptidases (KLKs) are a group of 15 serine proteases implicated in a variety of biological processes. Aberrant expression of *KLKs* has been associated with the development of certain cancers. However, the role of KLKs in salivary tumors has not been extensively studied. This study evaluated the expression of *KLKs* in both adenoid cystic carcinoma (ACC) and normal salivary gland tissue. We isolated total RNA from 39 formalin-fixed, paraffin-embedded samples, which included 24 ACCs and 15 normal salivary gland tissues. Complementary DNA, synthesized by reverse transcription, was combined with gene specific kallikrein primers (*KLK1–KLK15*) to allow for quantitative real-time PCR. Data was normalized to a β-actin housekeeping gene. Relative quantification analysis was performed using the ΔCq method. *KLK1–KLK15* expression was observed in both tissue types. However, *KLK1*, *KLK8*, *KLK11*, and *KLK14* were found to be downregulated in ACC. We propose that this may represent a multi-parametric panel providing diagnostic and prognostic information.

Keywords Kallikreins · Serine protease · Adenoid cystic carcinoma · RT-PCR · Gene expression

Introduction

Salivary neoplasms can arise in any of the three-paired major salivary glands or minor salivary glands of the oral cavity or oropharynx. In general, 70% of all salivary tumors occur in major salivary glands and fewer than 30% of salivary tumors occur in minor salivary glands [1]. Salivary neoplasms generally arise from ductal or secretory cells. Potential tumor cells of origin include myoepithelial cells, or cells from acini, intercalated ducts or striated intralobular ducts. Some salivary tumors are composed of only one cell-type while others may be composed of secretory, myoepithelial and ductal cells [2]. Adenoid cystic carcinomas (ACC), one of the more common malignant salivary neoplasms, are a mixture of both myoepithelial cells and ductal cells [2].

Peak incidence for ACC is in the fifth and seventh decades of life, but it may occur at any age, without clear sex predilection [3, 4]. Approximately 50–60% of ACC occur within minor salivary glands, most commonly in the palate. ACC has a slow growth rate; with time, the tumor becomes fixed and indurated, but this may not occur until late in the course of the disease [2]. Distant metastasis by hematogenous dissemination often occurs with disease progression but lymphatic spread remains rare [3]. Overall, metastasis occurs in up to 40% of patients [5]. Three major histomorphologic growth patterns are recognized: cribriform, tubular, and solid [2, 6]. All three patterns have a tendency for perineural invasion—this appears to be responsible for high local recurrence rates seen following surgery [1]. The treatment of choice for adenoid cystic carcinomas is aggressive surgical resection [3, 7]. The use of adjuvant radiation has also proved effective in the management of ACC [1]. However, there is still debate about whether radiation therapy should be given to all patients [6].

Early detection of cancer improves patient outcomes. Unfortunately, the diagnosis of ACC can be challenging and often occurs after the tumor has spread and invaded the perineural space of adjacent nerves. Recent research has focused on the identification of molecular markers and/or hormonal receptor expression in these tumors. A translocation involving *MYB* and *NFIB* genes has been described in ACC, with variable frequency and prevalence, and as yet unknown prognostic significance [8, 9].

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Kallikreins are expressed in nearly all tissues throughout the human body [10]. However, their relative abundances vary depending on the tissue type [11]. Kallikreins regulate tissue function and cell signaling through five distinct mechanisms [12]. These mechanisms include the generation or inactivation of polypeptide agonists from precursor proteins, the release of membrane-tethered growth factor receptor agonists, the activation or inactivation of growth factor receptors, signaling via the extracellular matrix or integrins, and the activation or inactivation of G protein-coupled proteinase-activated receptors (PARs) [12].

Aberrant *KLK* expression in malignant tissues suggests that kallikreins are involved in tumorgenesis [13]. One of the most studied cancer biomarkers is kallikrein-related peptidase (KLK) 3, better known as prostate-specific antigen (PSA). The clinical application of KLK3, as a biomarker, highlights the potential clinical utility of KLKs in the diagnosis and monitoring of tumors and their potential for recurrence. Research has shown KLKs contribute to the dissemination of cancer cells through their effects on cell migration and tissue invasion [14]. It is well documented that KLKs have proteolytic activity against extracellular matrix (ECM) proteins, cell membrane bound receptors, cell adhesion proteins, and growth factors and signaling molecules [15, 16]. The role of KLKs in salivary tumors has not been extensively studied, and thus, may further our understanding of the pathogenesis and clinical behavior of ACC.

This study aimed to analyze the expression of KLKs in adenoid cystic carcinomas and normal salivary tissue using real-time quantitative PCR (RT-qPCR).

Materials and Methods

Tissue Specimens

Formalin-fixed, paraffin-embedded tissue specimens for both adenoid cystic carcinoma and normal salivary tissue were obtained from the archives of the Division of Oral Pathology, Schulich School of Medicine and Dentistry, Western University and London Health Sciences Centre. Corresponding hematoxylin and eosin (H&E) stained slides were reviewed by a graduate student and a senior oral pathologist to confirm the diagnoses. Only specimens where the tissue of interest (e.g., either ACC or normal salivary tissue) encompassed the majority of the sample were selected. Any samples containing areas of inflammation were excluded. In total, 24 adenoid cystic carcinomas and 15 normal salivary tissues were deemed suitable for the study. Tables 1, 2 and 3 list patient and control demographic information.

H&E slides for ACC and normal salivary tissue were examined to identify areas consistent with the histopathological diagnosis. Areas were circled using a marker and transcribed onto corresponding areas on the FFPE blocks. 10 µm thick serial sections were cut from each FFPE block; specimens were then separated from surrounding areas along the scalpel lines and placed into 1.5 mL collection tubes.

Table 1 Demographic information, patterns and levels of altered kallikrein-related peptidases in minor salivary gland adenoid cystic carcinoma

Adenoid cystic carcinoma cases—minor glands										
Case number	Age	Sex	Location	Pattern			Comparison with levels in normal glands			
				C	T	S	KLK1	KLK8	KLK11	KLK14
1.	72	F	Palate	Y	Y	N				
3.	79	F	BM	Y	Y	N				
5.	58	M	Palate	Y	Y	N				
6.	56	M	Palate	Y	Y	N				
7.	66	F	Palate	Y	Y	N	High	High	High	High
8.	67	M	Palate	Y	Y	N				
9.	53	F	BM	Y	Y	N				
10.	58	F	Palate	Y	Y	N				
11.	76	F	Tuberosity	Y	Y	Y				
13.	77	F	BM	Y	N	N				
15.	58	F	BM	Y	Y	Y				
16.	47	M	Upper lip	Y	Y	N	High	High	High	High
19.	68	M	Maxillary sinus	Y	Y	N	High	High	Equal to normal	High
22.	45	M	Upper lip	Y	Y	N				
23.	79	F	BM	Y	N	N				

BM buccal mucosa, *C* cribriform pattern, *T* tubular/ductal pattern, *S* solid pattern, *Y* yes, present, *N* no, absent, *KLK* kallikrein-related peptidase

Table 2 Demographic information, patterns and levels of altered kallikrein-related peptidases in major salivary gland adenoid cystic carcinoma

Adenoid cystic carcinoma cases—major glands										
Case number	Age	Sex	Location	Pattern			Comparison with levels in normal glands			
				C	T	S	KLK1	KLK8	KLK11	KLK14
2.	53	F	SMG	Y	Y	N				
4.	67	F	Parotid	Y	Y	Y				
12.	73	F	Parotid	Y focal	Y	Y				
14.	33	F	Parotid	Y	Y	N				
17.	75	M	SMG	Y	Y	N				
18.	67	M	SMG	Y	Y	N				
20.	86	F	SL	Y	Y	N				
21.	13	M	SMG	Y	Y	N				
24.	53	F	SMG	Y	Y	Y		High		High

SMG submandibular gland, *SL* sublingual gland, *C* cribriform pattern, *T* tubular/ductal pattern, *S* solid pattern, *Y* yes, present, *N* no, absent, *KLK* kallikrein-related peptidase

Table 3 Demographic information for controls

Normal salivary tissue specimens.			
Case	Age	Sex	Location
1.	75	Female	Gingiva
2.	38	Female	Lower lip
3.	53	Female	Lower lip
4.	20	Male	Lower lip
5.	24	Male	Buccal mucosa
6.	68	Female	Lower lip
7.	24	Female	Floor of mouth
8.	20	Female	Lower lip
9.	47	Female	Lower lip
10.	42	Female	Lower lip
11.	18	Male	Lower lip
12.	68	Male	Uvula
13.	55	Male	Lower lip
14.	33	Female	Lower lip
15.	58	Female	Palate

Deparaffinization of FFPE Specimens

Xylene was added to the collection tubes containing FFPE specimens, and tubes were shaken briefly using a vortex mixer before incubating for 2 min. Collection tubes were then spun at 12,000×*g* for 2 min. Supernatant was discarded and the deparaffinization steps were repeated. Samples were then added to absolute ethanol and 70% ethanol, mixing and centrifuging (12,000×*g* for 2 min) for each step. Separated supernatant was discarded, and collection tubes were spun an additional 20 s before all residual fluid was removed. The tissue pellet in each tube was dried in a heating block (15 min at 55 °C).

RNA Isolation from Deparaffinized Specimens

Total RNA was isolated from the deparaffinized specimens using the High Pure FFPE RNA Micro Kit (Roche Applied Sciences, Mannheim, Germany, Catalogue Number: 04823125001). Briefly, tissue lysis buffer containing 10% sodium dodecyl sulphate was added to the tissue pellets. Samples were centrifuged and digested with proteinase K for 3 h. RNA was isolated using High Pure RNA binding spin columns.

Synthesis of cDNA from Isolated RNA

The isolated RNA was transcribed into complementary DNA (cDNA), via reverse transcription, using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, Catalogue number: 1708890).

Real Time Polymerase Chain Reaction (RT-PCR)

Real-time quantitative PCR reactions were carried out in 96 well plates using the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Each custom PCR plate accommodated six specimens and came loaded with KLK (KLK1–KLK15) and β-actin primer assays from Qiagen. The catalog numbers for the primer assays are listed in Table 4.

Synthesized cDNA (20 µL) was mixed with 225 µL of SYBR Green qPCR Master Mix and 205 µL H_2O. A volume of 20 µL was then pipetted from the reaction mix and added to the appropriate well on a PCR plate. The RT-qPCR protocol is briefly summarized as follows: denaturation for 10 min at 95 °C, 75 cycles of amplification for 15 s at 95 °C and 1 min at 60 °C. The specificity of PCR amplification was verified by post RT-qPCR melting curve analysis and

Table 4 Primer assays from qiagen used for RT-qPCR and melting temperature for *β-actin* and *KLK1–15* primers

Primer assays from qiagen used for RT-qPCR		Melting temperature for *β-actin* and *KLK1-15* primers	Number of base pairs per primer
Primer assay	Catalog number	T_m values (±0.5 °C)	
β-actin	QT01680476	85.5	
KLK1	QT00020664	82.5	118
KLK2	QT00088466	82.5	134
KLK3	QT00027713	84.5	101
KLK4	QT00495159	83.5	172
KLK5	QT00010437	79.5	96
KLK6	QT00013972	81.5	146
KLK7	QT00028343	84.5	171
KLK8	QT00017689	82.5	114
KLK9	QT00057190	85.5	184
KLK10	QT00039816	82.0	134
KLK11	QT00011011	80.5	183
KLK12	QT00067977	81.0	81
KLK13	QT00029876	85.0	185
KLK14	QT00039928	85.0	104
KLK15	QT00035735	84.0	135

agarose gel electrophoresis. Data was normalized to β-actin as a control gene.

Melting Curve Analysis of PCR Products

The generated melting curves were analyzed for each PCR product. Table 4 lists the known melting temperatures (T_m) for *KLK1–KLK15* and *β-actin*. These values were compared to the generated melt peak T_m values. Peaks with a T_m value differing from the known values, or melting curves showing primer dimer formation were either excluded or interpreted as showing no expression.

Table 5 Percentage of ACC and normal salivary tissues expressing *KLK1–KLK15* and two-tailed p-values from the comparison of ΔCq values between ACC and normal salivary (Mann–Whitney *U* test)

Gene	ACC	Normal salivary tissue	Two-tailed *p* value
KLK1	73.0	84.6	0.0198
KLK2	82.0	69.2	0.1061
KLK3	68.0	84.6	0.2645
KLK4	64.0	61.5	0.4525
KLK5	82.0	84.6	0.0922
KLK6	68.0	76.9	0.3383
KLK7	64.0	69.2	0.5571
KLK8	82.0	76.9	0.0400
KLK9	45.0	38.5	0.5135
KLK10	73.0	69.2	0.2756
KLK11	68.0	84.6	0.0064
KLK12	86.0	100	0.0655
KLK13	41.0	61.5	0.1996
KLK14	91.0	84.6	0.0440
KLK15	77.3	69.2	0.1382

Agarose Gel Electrophoresis of PCR Products

The products of PCR were visualized using agarose gel electrophoresis. 100 mL of running buffer (20 mL of TAE [50×] to 1000 mL of distilled H_2O) was added to a flask containing 2 g of Agarose PCR Plus. The mixture was repeatedly heated and swirled until it became clear; ethidium bromide was added to the gel before it was brought to a boil and then allowed to cool to 60 °C. At this time the gel was poured into a casting tray containing a single comb, creating 20 wells, and was allowed to cool further before removing the comb. The casting tray was inserted into the Owl EasyCast B2 Mini Gel Electrophoresis System (Thermo Fisher Scientific, Asheville, NC, USA) with the wells positioned closest to the cathode. The chamber was filled with running buffer, fully covering the gel. The first well was loaded with 12 µL of TrackIT 100 bp DNA Ladder, and the next 15 were loaded with 12 µL of amplified *KLK1* through *KLK15* PCR products, which each contained 1 µL of DNA Gel Loading Dye [6×]. The chamber was then connected to power source, and electric current was applied to allow for adequate migration at which time the gel was placed on an ultraviolet viewing box to capture digital photos.

Statistical Analysis

The ΔCq method was used to analyze the RT-qPCR data. The ΔCq values were calculated using the formula $\Delta Cq = 2^{Cq(\beta\text{-actin}) - Cq(KLK)}$. The particular *KLK* with the

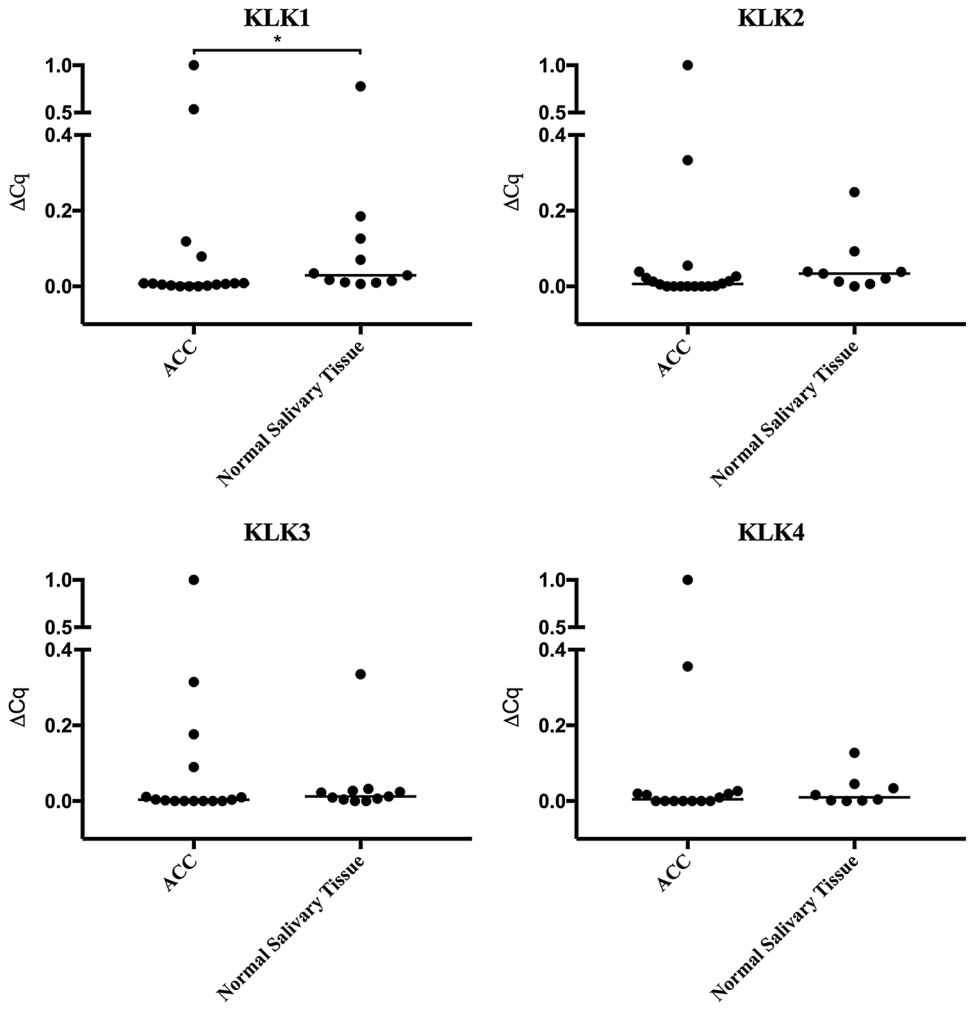

Fig. 1 Scatter plots of ΔCq values for KLK1–4 in ACC and normal salivary tissue. Statistical comparison using Mann–Whitney *U* test. *p ≤ 0.05; **p ≤ 0.01

lowest Cq value was assigned a ΔCq value of 1, and used as a calibrator to convert ΔCq values for remaining specimens. Calculated relative quantities for each *KLK* were compared between ACC and normal salivary tissue to look for differences in expression. The Mann–Whitney *U* Test was used for statistical analysis, with a level of significance set at 0.05 ($p < 0.05$).

Results

Expression of KLK by Tumor Pattern and Location

In total, 15 ACC arose in intraoral and maxillary sinus glands, and 9 arose in major salivary glands (Tables 1, 2). Two ACC in minor glands had a solid growth pattern, while all showed a cribriform pattern and most had a tubular pattern. Three ACC in minor glands were outliers, expressing higher than or equal levels of, *KLK*s 1, 8, 11 and 14 as normal salivary tissues. Three ACC in major glands had a solid growth pattern, and all nine contained cribriform and tubular growth patterns. Only one ACC of major glands showed higher levels of *KLK*s 8 and 14 than normal tissues. As all tissue histological variants were contained in a tested sample, correlation of *KLK* expression with individual patterns could not be determined.

Specimens Expressing KLK cDNA in ACC and Normal Salivary Tissue

The expression of kallikreins (*KLK1–KLK15*) was examined in ACC and normal salivary tissue using RT-qPCR. As shown in Table 5, all members of the KLK family were expressed in both ACC and normal salivary tissue. Three of the ACC specimens and two of the normal salivary tissue specimens were excluded from the study following the review of their melting curves due aberrant β-actin amplification, which was reflected in discrepancies between the expected and observed melt peak T_m values.

KLK9 was the kallikrein least likely to be detected in normal salivary tissue and it was found in only 38.5% of

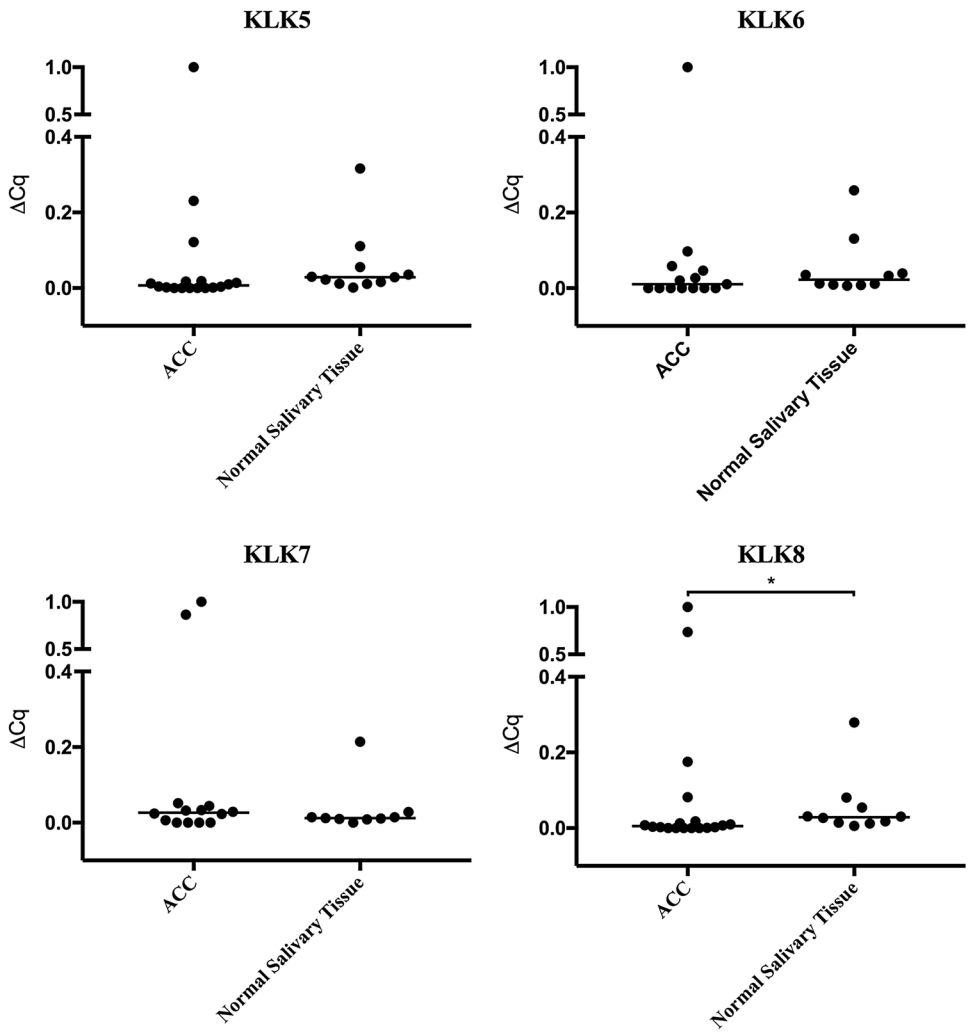

Fig. 2 Scatter plots of ΔCq values for KLK5–8 in ACC and normal salivary tissue. Statistical comparison using Mann–Whitney *U* test. *p ≤ 0.05; **p ≤ 0.01

samples. In ACC, *KLK9* and *KLK13* were both expressed in a smaller number of specimens compared to the other kallikreins. The percentage of ACC samples expressing *KLK9* and *KLK13* was 45% and 41%, respectively. Both *KLK12* and *KLK14* were found to be widely present in both tissue types.

Comparison of KLK cDNA Expression Between ACC and Normal Salivary Tissue

Differences in kallikrein cDNA expression in ACC and normal salivary tissue were compared using the ΔCq method. Figures 1, 2, 3, and 4 depict the specific ΔCq values from each specimen for each *KLK*. The data are arranged in scatter plots with a horizontal line representing the median ΔCq value.

KLK1, *KLK8*, *KLK11*, and *KLK14* were all found to have significantly lower ΔCq values in ACC compared to normal salivary tissues. In contrast, no statistical difference in ΔCq values was seen for *KLK2–KLK7*, *KLK9*, *KLK10*, *KLK12*, *KLK13* and *KLK15*. The calculated two-tailed p-values are presented in Table 5.

For *KLK1*, *KLK8*, *KLK11*, and *KLK14*, median and quartile ΔCq values for both tissue types are presented in Table 6 and Fig. 5. In the box and whisker plots, all ΔCq values are shown, the median values are marked by a horizontal line within the boxes, which extend from the 25th to 75th percentiles, and the whiskers extend from minimum to maximum values.

Verification of PCR Products on Agarose Gel Electrophoresis

Amplified PCR products were analyzed by agarose gel electrophoresis for the identification of *KLK1–15* assays.

The four gels shown in Fig. 6 confirm good primer specificity. In Fig. 7, the gel has been converted to gray scale to allow easier comparison to the DNA ladder. Careful inspection of the separated bands in the gel, with

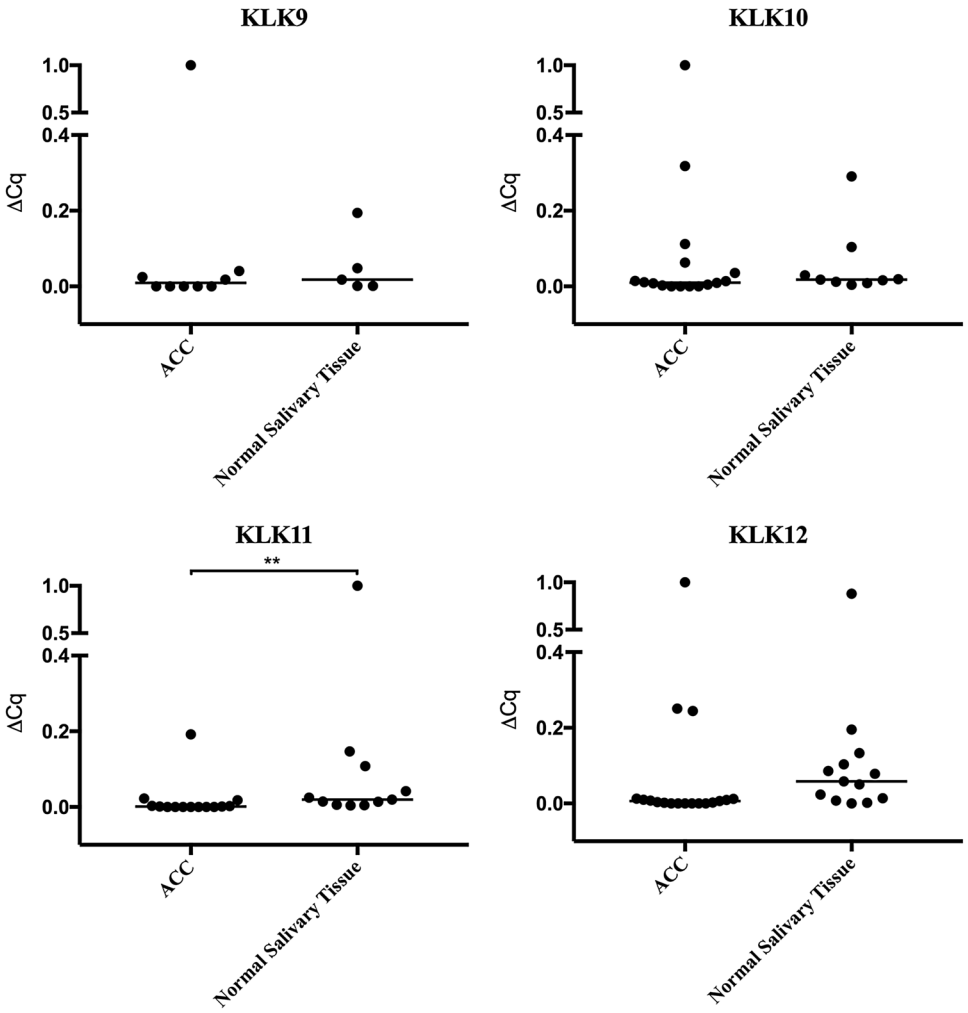

Fig. 3 Scatter plots of ΔCq values for KLK9–12 in ACC and normal salivary tissue. Statistical comparison using Mann–Whitney *U* test. *p ≤ 0.05; **p ≤ 0.01

reference to Table 4, confirms the presence of *KLK1–15* and ultimately the methodology of cDNA synthesis from RNA isolated from FFPE specimens.

Discussion

This study confirms that all human kallikreins are expressed in normal salivary tissues. However, to our knowledge, this is the first study to explicitly exclude samples with evidence of inflammation. This is important given that previous studies have implicated the involvement of kallikreins in inflammatory processes, and histological inflammation can affect KLK expression levels [11, 17]. Additionally, this is the first study describing kallikrein expression in normal salivary tissue and adenoid cystic carcinoma using RT-qPCR. A correlation between location, tumour pattern and *KLK* expression could not be made, and there were no apparent differences between the outliers in minor glands although one of the outliers did arise in the maxillary sinus.

In this study, we have demonstrated decreased expression of *KLK8* in ACC compared to normal salivary tissues. Previous work in our laboratory showed differences in immunoexpression levels of ACCs compared to normal controls to be insignificant [18]. Contrary to growing evidence, we observed decreased KLK8 mRNA levels with unchanged protein levels of KLK8 [18, 19]. A possible explanation for this could be that the immunoperoxidase staining technique used by Darling et al. was not sensitive enough to detect subtle differences between specimens. KLK8 is a favourable prognostic marker—it is known to suppress cancer metastasis, and is indicative of improved overall survival

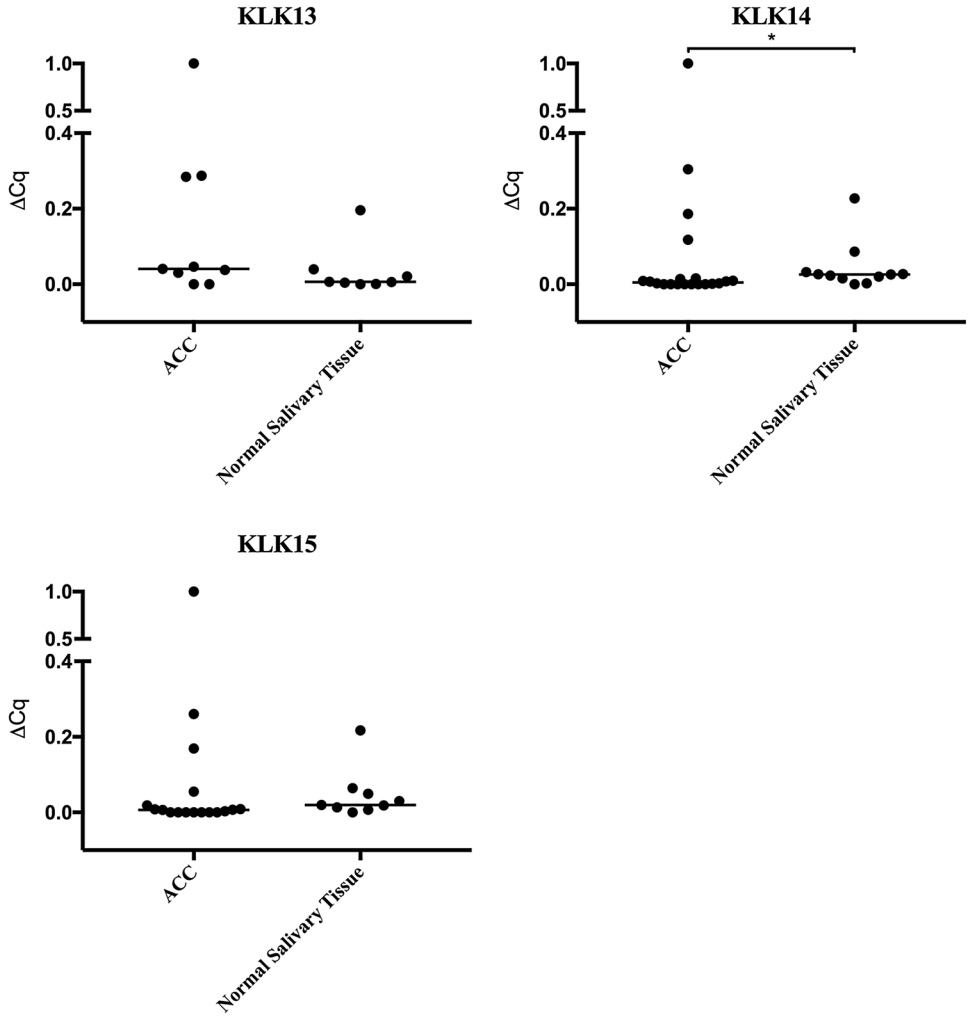

Fig. 4 Scatter plots of ΔCq values for KLK13–15 in ACC and normal salivary tissue. Statistical comparison using Mann–Whitney *U* test. *p ≤ 0.05; **p ≤ 0.01

Table 6 Comparison of median and quartile ΔCq values in ACC and normal salivary tissue for *KLK1*, *KLK8*, *KLK11*, and *KLK14*

Gene	Tissue type	Median ΔCq	First ΔCq quartile (Q1)	Third ΔCq quartile (Q3)
KLK1	ACC	0.00710	0.00225	0.02611
	Normal salivary tissue	0.02903	0.01288	0.09830
KLK8	ACC	0.00543	0.00013	0.01682
	Normal salivary tissue	0.02862	0.01543	0.04847
KLK11	ACC	0.00146	0.00000	0.01030
	Normal salivary tissue	0.01959	0.00978	0.07486
KLK14	ACC	0.00467	0.00014	0.01420
	Normal salivary tissue	0.02583	0.01790	0.02944

[20–22]. Additionally, *KLK8* downregulation may facilitate hematogenous spread through VEGF upregulation [20]. We could speculate that the *KLK8* downregulation eliminates its ability to suppress tumor cell invasion and may explain ACC's propensity for perineural spread.

KLK14 expression is under steroid hormone regulation, and it has a restricted expression pattern [23]. Previously published results from our laboratory found the immunoexpression levels of KLK14 in ACCs were higher, though not significantly, relative to normal control tissue [24]. In this study, we have found that protein levels seem to be upregulated and gene expression is downregulated for *KLK14* in ACC. It can be hypothesized that KLK14 may inhibit its own transcription in a negative feedback loop as it becomes more abundant. To our knowledge, a negative feedback loop that relates to kallikrein transcription has yet to be described, although it is known that they have a hormone like function [12]. Further studies are needed to either confirm or dispute its existence.

The mRNA and protein concentrations of KLK1 and KLK11 in normal tissues have been studied previously, however this study confirmed their presence in salivary gland tumors for the first time. *KLK1* and *KLK11* have been found to be downregulated in ACC compared to normal

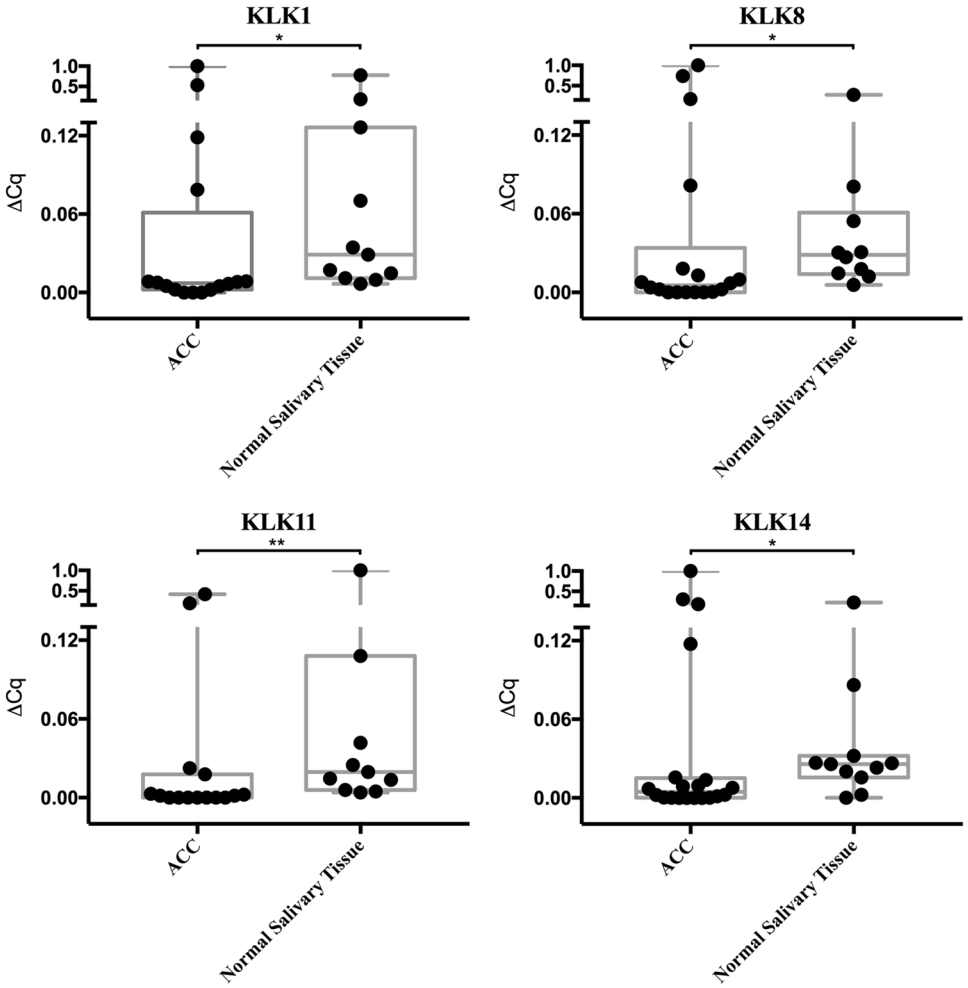

Fig. 5 Box and Whisker plots of ΔCq values for KLK1, KLK8, KLK11, and KLK14 in ACC and normal salivary tissue. Statistical comparison using Mann–Whitney *U* test. *p ≤ 0.05; **p ≤ 0.01

salivary tissues. The parallel co-expression of *KLK1* and *KLK11* is an uncommon finding, and has not previously been documented in salivary gland tumorigenesis.

Studies examining *KLK1* have suggested that single nucleotide polymorphisms (SNPs) of kallikrein genes possess value as putative genomic biomarkers [13]. The presence and/or significance of kallikrein SNPs in salivary gland tumors have not been studied to date, however it is possible that they may prove to be useful biomarkers in future studies.

Previously, Darling et al. have suggested that individual kallikreins cannot be considered as specific markers for salivary gland tumors [25–27]. Furthermore, if KLKs are to be useful as biomarkers in salivary gland tumors multiparametric panels will need to be identified [24]. In this study, we have documented for the first time changes in co-expression of multiple *KLKs* in a salivary gland tumor. It is possible that the downregulation of *KLK1*, *KLK8*, *KLK11*, and *KLK14* may represent a newfound KLK panel for the diagnosis of ACC, and this warrants further investigation.

This study is the first to quantify kallikrein expression in ACC and normal salivary tissue from FFPE extracts. The expression of individual kallikreins was normalized to β-actin in each sample and inter-specimen analysis was then performed using relative quantification, although this overlooks the fact that the measured RNA levels may not reflect the actual expression in tissues before fixation [28]. Therefore, if our study used fresh tissues rather than fixed tissues, it is possible that the ΔCq values would be higher in both ACC and normal salivary tissue, and that the absolute difference in ΔCq values between the two would be larger for *KLK1*, *KLK8*, *KLK11*, and *KLK14*. If this is true, the accuracy of a future multiparametric panel for ACC may be improved if it is developed using fresh tissue. In any event, this also warrants further investigation.

Future studies should aim to look at expression levels of *KLK1, KLK8, KLK 11,* and *KLK14* in both saliva and tissue specimens in patients with ACC in order to further investigate their use for cancer detection and diagnosis.

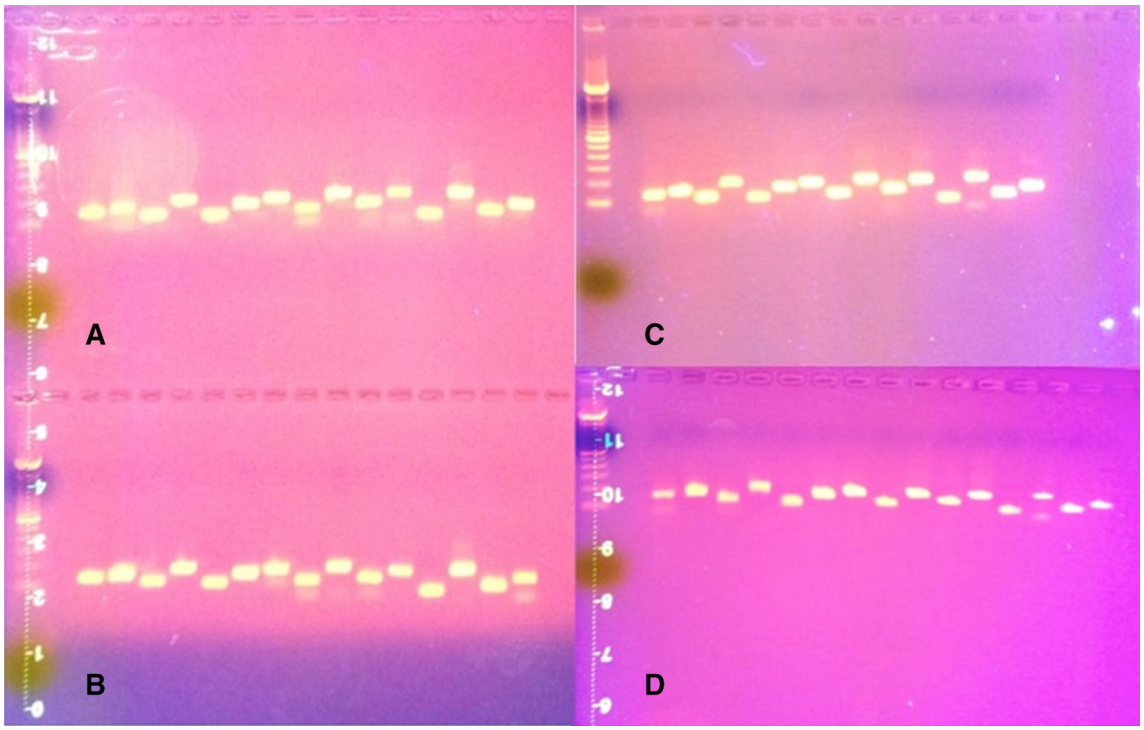

Fig. 6 Agarose gel electrophoresis of PCR products for the primers KLK1–15 in ACC (**a**–**c**) and normal salivary tissue (**d**)

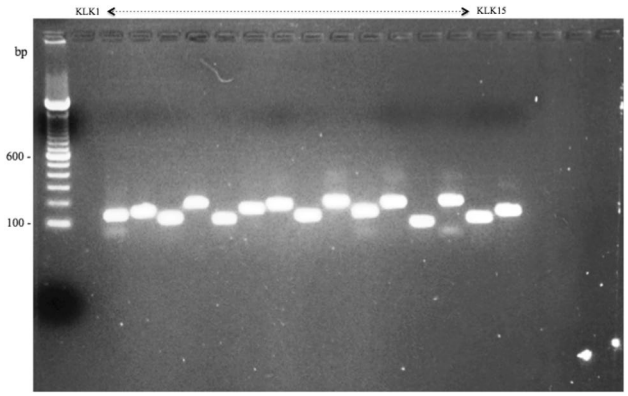

Fig. 7 Agarose gel electrophoresis of PCR products from ACC showing band separation of KLK1–15 primers in reference to TrackIT 100 bp DNA ladder

Conclusion

In this study, we looked at the gene expression of kallikreins in ACC and normal salivary tissues. For the first time, we have quantified the expression of *KLK1–KLK15* in both tissue types using RT-qPCR. Furthermore, the extraction of total RNA from archived FFPE samples is also novel to the study of *KLK* expression in salivary gland tissues. We were able to confirm successful amplification of the *KLK* PCR products using melting curve analysis and gel electrophoresis.

Our results show that all *KLKs* are expressed in both ACC and normal salivary gland tissue. Furthermore, we report differences in the levels of certain *KLKs* in ACC compared to controls. Specifically, the expression of *KLK1*, *KLK8*, *KLK11* and *KLK14* are downregulated in ACC.

We believe this study suggests that aberrant kallikrein expression may be involved the development of ACC and may contribute to its distinct clinical behavior, which ranges from local invasion to distant metastasis. We hope to further investigate whether a multiparametric panel of *KLK1*, *KLK8*, *KLK11* and *KLK14* may be useful in the diagnosis or related to prognosis of ACC, and whether tumor pattern and location correlates with kallikrein expression.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Western University Research Ethics Board, Study Number 103783 and with

the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

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