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Expression of Kallikrein-Related Peptidases (KLKs) in Adenoid Cystic Carcinoma

Zachary R. Kerr
The University of Western Ontario

Supervisor
Dr. Mark Darling
The University of Western Ontario Joint Supervisor
Dr. Zia Khan
The University of Western Ontario

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Abstract

Adenoid cystic carcinoma (ACC) of salivary glands is a malignancy known for its slow growth pattern, but poor long-term survival despite aggressive treatment. This highlights the need for an improved approach to its management.

It has been suggested that kallikrein-related peptidases (KLKs), a group of 15 serine proteases, may serve as biomarkers for salivary gland tumors. KLKs are involved in numerous biological processes and have been linked to several cancers.

This study evaluated *KLK* gene expression in ACC and normal salivary gland tissue (NSGT). Total RNA was isolated from 40 formalin-fixed, paraffin-embedded samples, which included 25 ACCs and 15 NSGTs. RNA samples were subjected to reverse transcription and RT-qPCR utilizing human-specific *KLK1-15* primers. *KLK1-KLK15* expression was observed in both ACC and NSGT. Comparative ΔCq analysis found *KLK1*, *KLK8*, *KLK11*, and *KLK14* to be downregulated in ACC. This may represent a multiparametric panel for its diagnosis; however, additional studies are needed.

Dedication

I dedicate this thesis to baby Marcus.

Acknowledgments

I not only want to acknowledge, but sincerely thank all that made this thesis possible. I am grateful to Drs. Tom Daley, Mark Darling and Zia Khan for their guidance and unwavering patience. Their willingness to work around my schedule will not be forgotten. I would like to thank Linda Jackson-Boeters for her technical assistance throughout the project. She was truly an invaluable resource. I am thankful to my wonderful parents. They have always encouraged me to find happiness, showed me the value of hard work, and been there to share in my accomplishments. Lastly, I wish to thank my brilliant and beautiful wife Nora. She has been instrumental in navigating me through this journey and has always been a shoulder I can count on. I could not have done this without her.

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List of Abbreviations

ACC: Adenoid Cystic Carcinoma

ACS: American Cancer Society

AUA: American Urological Association

cDNA: Complementary DNA

Cq: Quantification Value

CT: Computed Tomography

DNA: Deoxyribonucleic Acid

ECM: Extracellular Matrix

EDTA: Ethylenediaminetetraacetic Acid

EMT: Epithelial-Mesenchymal Transition

FFPE: Formalin-Fixed, Paraffin-Embedded

FNA: Fine-Needle Aspiration

H&E: Hematoxylin & Eosin

KLK: Kallikrein-Related Peptidase Protein

KLK: Kallikrein-Related Peptidase Gene

miRNA: MicroRNA

MRI: Magnetic Resonance Imaging

mRNA: Messenger RNA

NSGT: Normal Salivary Gland Tissue

PAR: Protease-Activated Receptor

PCR: Polymerase Chain Reaction

PLCO: Prostate, Lung, Colorectal, and Ovarian

PSA: Prostate-Specific Antigen

RNA: Ribonucleic Acid

RT-qPCR   Real-Time Quantitative Polymerase Chain Reaction

SNP: Single Nucleotide Polymorphism

T_m Melting Temperature

uPA   Urokinase-Type Plasminogen Activator

Chapter 1

1   Introduction

1.1   Salivary Neoplasms

Tumors originating from salivary gland tissue can be derived from either

parenchymal epithelial cells or mesenchymal stroma (1). However, in the vast majority

of cases, they are of epithelial origin (2). These epithelial tumors typically arise in adults

unlike their mesenchymal counterparts, which are most common amongst children (1).

The mesenchymal tumors are usually benign. They may be of vascular origin, or contain

populations of fibroblasts and histiocytes due to fibrohistiocytic differentiation (1). In

contrast, the classification of primary epithelial neoplasms is more challenging due to the

immense diversity of the tumors and the paucity of their occurrence (3). It is estimated

that the annual incidence of salivary gland tumors worldwide ranges from 1.0 to 6.5 cases

per 100,000 people (3). Therefore, in a city like London, Ontario, with an estimated

population of 366,151 (4), one could expect to see anywhere between 3 to 24 newly

diagnosed cases per year. Fortunately, most of these cases will be benign.

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, the major salivary

glands account for over 70% of all salivary tumors (5), and less than 30% of salivary

tumors are said to occur in minor salivary glands (1). Furthermore, knowing the site of

origin can aid in the prediction of whether the tumor is likely to be benign or malignant.

Typically, salivary tumors occurring in the sublingual gland or the minor salivary glands

are more likely to be malignant compared to those occurring in the larger submandibular

or parotid glands (3). Minor salivary gland tumors, which are most commonly found on

the palate, followed by the upper lip and buccal mucosa, account for approximately 25% of all tumors (2). Overall, 50% of these tumors will prove to be malignant, with certain high-risk sites such as the tongue, retromolar region and floor of the mouth approaching 90% (1). Keeping with this trend, tumors of the sublingual gland, the smallest of the major salivary glands, have a high likelihood of being malignant, up to 90% (2). Luckily, they account for no more than 1% of all salivary tumors (3). Neoplasms arising from the submandibular gland, the next largest gland, represent roughly 10% of all salivary tumors (2, 5). Of these, the frequency of malignancy is approximately 40% (2). Lastly, the parotid gland, which is the largest salivary gland, is the most common site to find a salivary neoplasm. Fortunately, most of these tumors are benign. According to Neville et al., it can be stated that two-thirds to three-quarters of salivary tumors occur in the parotid gland, and two-thirds to three-quarters of these parotid tumors are benign (3).

Salivary neoplasms, independent of their site of origin or whether they are benign or malignant, generally arise from either the ductal or secretory cells of a salivary gland. More specifically, potential tumor cells of origin include myoepithelial cells, or those from acini, intercalated ducts or striated intralobular ducts (1). Stem cells, or reserve cells as they are more commonly referred to, are also found within salivary glands. Given their undifferentiated nature, many believe that these cells are the cells of origin for most observed salivary tumors (2). Regardless of whether or not this is true, the line of differentiation ultimately determines the tumors histopathological pattern and how it will be classified. Some salivary tumors are composed of only one cell type while others may be composed of secretory, myoepithelial and ductal cells (1). For example, adenoid

cystic carcinomas (ACCs) are a mixture of both myoepithelial cells and ductal cells (2, 3).

1.1.1 Adenoid Cystic Carcinoma

ACC is one of the most common malignant salivary neoplasms (3). It is also one of the most easily recognizable given its characteristic histologic pattern, often referred to as "Swiss cheese" (3).

1.1.1.1 Clinical Features

The peak incidence of ACC is noted within the fifth and seventh decades of life (2), but may occur in people of any age (1, 6). They can be found in both men and women, with no clear sex predilection (2, 3, 6). Most ACCs occur within minor salivary glands, approximately 50-60% of the time, with the palate being the site predominantly affected (3). In fact, they are the most common salivary malignancy of the palate (7). These tumors can also arise within the major salivary glands. The parotid gland is affected most (1, 2), but given its propensity for benign tumors, ACCs are infrequently seen there (3). It is estimated that they comprise only 2 to 3% of all parotid gland tumors (3). In comparison, ACCs are the most common malignancy of the submandibular gland (3), even though a smaller percentage of the total number of tumors are found there.

ACC is known for its slow growth rate (1-3). With time the tumor becomes fixed and indurated (1), but this may not occur until late in the course of the disease. Pain often precedes any noticeable mass, and it may be the only reason a patient seeks treatment (3). The pain is often described as a persistent dull ache (3). Given ACC's propensity for neural involvement, facial nerve weakness or paralysis may be the initial presenting symptom in some patients with a parotid tumor (2). Common features of late-stage

palatal tumors are evidence of mucosal ulceration or bone destruction on radiographs (3).

Palatal lesions are also capable of perineural spread, usually along the palatine branches

of the maxillary nerve (7). Distant metastasis by hematogenous dissemination often

occurs with disease progression (6). However, lymphatic spread remains rare (6).

Overall, metastasis occurs in up to 40% of patients (8). The sites most commonly

affected are the lungs followed by bone (6, 8).

1.1.1.2   Histopathology

Three major histomorphologic growth patterns are recognized (1-3). They

include cribriform, tubular, and solid (5). Most tumors show evidence of all three

patterns, but one pattern typically dominates (3). Identifying the predominant pattern is

important, as it will dictate tumor behavior and aggressiveness (5).

The cribriform pattern has the prototypical Swiss cheese microscopic appearance.

Multiple cyst-like spaces, containing a basophilic mucoid material, an eosinophilic

hyalinized material, or a combination of both, are observed, and stain positive for mucin

(1, 3). Oval nests of small cuboidal epithelial cells with hyperchromatic nuclei surround

these spaces (1). Theses cuboidal cells have scant cytoplasm, rarely contain mitotic

figures and are usually homogenous in appearance (3). The tumor is often embedded in a

mature, hyalinized stroma (1, 3).

The tubular pattern is composed of similar appearing tumor cells, but the nests are

smaller (2). One to three layers of epithelial cells usually surround distinct duct-like

structures (1, 2). Occasionally, a layer of myoepithelial or ductal cells can be seen (3).

The solid basaloid pattern does not have a predisposition to forming ductal or

cystlike structures (3), but instead, the tumor cells arrange themselves in solid nests (1).

Furthermore, unlike the cribriform or tubular patterns, this configuration shows evidence of increased mitotic activity and cellular pleomorphism (3).

Although not pathognomonic of ACC, all three patterns have a tendency for perineural invasion. Microscopically, the tumor cells can often be seen wrapping themselves around nerve bundles (3). Involvement of the perineurium then allows the tumor to invade the surrounding lymphatic vessels (1). This facilitates distant metastasis, and ultimately, it is responsible for the high local recurrence rates seen following surgery (1).

1.1.1.3   Diagnosis

The assessment of a patient with a suspected salivary gland tumor, such as ACC, is no different than that of any other patient who may present with any number of different complaints. It begins by performing a comprehensive history and physical examination followed by appropriate imaging and tissue biopsy (9). Equipped with this information, a diagnosis and surgical plan can then be devised.

When the suspicious lesion involves a minor salivary gland, an open biopsy is the preferred method to arrive at a diagnosis. However, a mass involving a major salivary gland complicates the diagnostic workup. Currently, the optimal method to make this diagnosis remains controversial (9, 10).

When the mass involves the parotid gland, many surgeons will elect to perform fine-needle aspiration (FNA) (2). Despite its obvious benefits, such as low cost, easiness to perform and safety (10, 11), many have questioned its clinical usefulness due to poor sensitivity (12). A recent meta-analysis by Schmidt et al. assessed the diagnostic accuracy of FNA for parotid gland tumors (12). They were able to identify 64 studies,

involving 6,169 cases, which looked at the ability of FNA to correctly identify malignancy (12). They found the overall specificity to be quite high at 96%, but the sensitivity was much lower at only 79% (12). An earlier published retrospective cross-sectional study by Zbären et al. found similar findings. They reported the sensitivity and specificity of FNA in detecting malignant salivary tumors to be 74% and 88%, respectively (11). Furthermore, their results also showed that the histologic tumor type was correctly diagnosed by FNA only 35% of the time for malignant neoplasms (11). It has been suggested that inadequate tissue sampling is the most common reason for this diagnostic error, and the use of ultrasound-guidance may improve the accuracy of FNA (5). However, the accuracy is also undoubtedly influenced by the experience of the cytopathologist and the inherent difficulty of classifying malignant salivary neoplasms given their low volume and diverse nature (5, 11).

The treating physician should consider the limitations of FNA and ponder two key questions before making treatment decisions. First, is the sensitivity of FNA sufficient enough to exclude malignancy when the results are negative (12)? Second, can the results of a FNA, which is positive for malignancy, be used in determining the extent of surgery (12)? Unfortunately, no practice guidelines have been developed to definitively answer these questions. Therefore, it is the responsibility of the physician to know the shortcomings of FNA, such as its low sensitivity. It has been suggested that FNA has the potential to misguide clinicians, and its results have erroneously been used to plan the extent of surgery (10). Zbären et al. insist that surgical decisions, such as the need for an elective neck dissection or total parotidectomy, require knowledge of the tumors

histologic subtype and/or grade (11). Therefore, FNA should not be used in surgical

planning (11).

Computed tomography (CT) and magnetic resonance imaging (MRI) are also

used in the diagnostic workup of salivary gland masses. Imaging can be very helpful in

determining the anatomic location of a tumor and delineating its margins (5). It also

provides valuable information about the presence of any neurovascular involvement,

bony invasion, intracranial extensions or distant metastasis (5). Many clinicians believe

that MRI is the best modality to assess ACCs given their high rate of perineural spread

(13). It is felt that MRI is better able to demonstrate neural invasion or any infiltration of

the skull base (13). However, MRI, like CT, tends to underestimate the true extent of

ACCs (14).

The ability to make a specific diagnosis using imaging appears to have its

limitations. Ikeda et al. were unable to find any signs on MRI that would allow for

perfect sensitivity or specificity in diagnosing pleomorphic adenomas (15). This finding

is consistent with that of other researchers, and it has prompted some to suggest that

radiology should only be used for operative planning and not for diagnosis (10). To

complicate matters even further, ACCs can appear as either benign or malignant on

imaging (7).

1.1.1.4   Treatment and Prognosis

The treatment of choice for ACCs is aggressive surgical resection (6, 16). Given

that the hallmark features of this tumor are local recurrence, perineural spread, and late

distant metastasis, obtaining clear margins at the time or surgery is paramount (14). For

lesions of the parotid gland, realizing this goal may necessitate total parotidectomy (9)

and resection of the facial nerve, should it be infiltrated or encased by tumor (2, 5), When the tumor originates in the submandibular gland, special attention needs to be directed towards the lingual, hypoglossal and marginal mandibular nerves (5). If there is any evidence of neural involvement, the nerve in question should be sacrificed (5) during the sialoadenectomy procedure (1). Any ACC found intraorally requires wide local excision with a 1-cm margin and one uninvolved anatomic barrier (9). This may require radical bony excision to obtain tumor-free margins (2, 5).

ACC is radiosensitive (1, 17), and the use of adjuvant radiation has proved effective in the management of the disease (2). However, there is still debate about whether radiation therapy should be given to all patients. It has been suggested that surgery alone may be adequate when treating small tumors with clear margins (5, 17, 18). Some institutions have elected to provide radiation only in cases with unfavorable features such as positive resection margins (6, 19, 20), tumors in proximity to the cranial base (19), or those with a solid growth pattern (19, 20). However, there are advocates who recommend combined surgery and radiation in all patients (21, 22). Simpson et al. reported local control rates of 86% when adjuvant radiation was given compared to only 11% when it was withheld (23). Results published by The University of Texas MD Cancer Center showed 5 and 10-year local control rates of 95% and 86%, respectively, in patients who received surgery and postoperative radiotherapy (21). More recently, based on their case series and review of the literature, Chen et al. declared that combined modality therapy should be the standard of care (22). According to Chen et al., all resectable ACCs should be treated with surgery and adjuvant radiotherapy, of at least 60 Gy, to afford patients the best chance of survival.

Proponents against giving adjuvant radiation treatment indiscriminately to patients with ACC cite a lack of evidence to show it provides a clear survival advantage (19). Even if this is true, the utility of radiation should not be discounted, and the importance of improved local control cannot be overstated. One can postulate that a definitive survival benefit has yet to be shown because postoperative radiation is ineffective at preventing distant metastases (8, 22).

It is well established that patients with distant metastasis have worse survival outcomes (8). However, many patients live for remarkably prolonged periods after the development of distal disease (6, 8, 17). This is particularly true when there is no evidence of local disease (8, 17). Khan et al. found the median survival after the discovery of an isolated pulmonary lesion to be 6.8 years (17). Furthermore, researchers have also shown that distant metastasis occurs independently of local treatment outcomes (6, 17). Based on these observations one can argue that distant disease should not preclude treatment, and in fact, both local and distal disease should be treated as separate entities (6, 17).

Neck disease, due to lymphatic spread, is rare in ACC (17). As results, it is common belief that neck disease plays only a small role in overall survival outcomes (6). Therefore, neck dissections have not frequently been performed (17) and have been reserved only for cases with evidence of lymphatic spread (6, 14). However, recent studies have shown that the incidence of occult neck metastases is higher than previously thought (24, 25). A multicenter study by Amit et al., determined the incidence of occult nodal disease to be 17%, amongst 226 patients who underwent elective neck dissections

(24). This is similar to the results published by Lee et al.; they found an occult metastases rate of approximately 15% (25).

Head and neck surgeons accustomed to treating squamous cell carcinoma tend to follow the dogma that elective neck dissection should be performed when the risk of occult metastasis is greater than 15% (26). Therefore, practitioners who previously avoided elective neck dissections in patients with ACC, due to the held belief that regional disease is rare, have been forced to reevaluate their practices.

As one could expect, the topic has created much controversy (9, 25). Bell et al., after reflecting on their decades of clinical experience managing malignant salivary pathology, advocate for elective neck dissection in all patients with high-grade tumors, advanced local disease, symptomatic facial nerve involvement, and advanced age (27). However, they suggest that it is probably not necessary in small, low-grade tumors (27). Recently, Mesolella et al. stated that all first echelon nodes need removal in order to control for distant metastasis even if there is no evidence of nodal disease (28). Similarly, based on their finding of improved rates of neck control at 5 and 10 years in those receiving elective neck dissection, 98% and 98%, compared to those not receiving neck dissections, 95% and 89%, Balamucki et al. recommend the surgical removal of sentinel lymph nodes (29).

According to Neville et al. death usually results from either local recurrence or distant metastases (3). The overall prognosis of ACC is poor, although this may not be reflected in the 5-year survival rates (16). Survival rates at 5-years are approximately 70%, but fall to 50% at 10-years (6, 17). By 15 years, fewer than 35% of patients are still alive (6, 17). This trend highlights the importance of long-term follow-up. Local

recurrence and distant metastasis have both been observed 15-years after the initial presentation of the primary tumor (6). Researchers have attempted to identify factors that can help predict in which patients this is most likely to occur.

Factors associated with poorer outcomes include positive resection margins at the initial time of surgery (6, 14, 21, 22), advanced stage of the primary tumor (6, 17, 19), and tumors with a solid histopathologic growth pattern (8, 19).

Szanto et al. in 1984 established a histologic grading system for ACC based on the degree of the solid component (30). Tumors containing no solid component are grade 1, tumors containing less than 30% solid component are grade 2, and tumors with greater than 30% solid component are grade 3 (30). The histologic subtypes composed of the solid pattern have been found to be a poor prognostic factor in patients previously treated for ACC (30). The 15-year survival rates for grade 3, grade 2 and grade 1 tumors are 5%, 26% and 39%, respectively (30). Franzen et al. also found that ACCs composed of partly solid histologic features have worse patient outcomes compared to those tumors lacking solid areas (31). Furthermore, they found a correlation between DNA aneuploidy and tumors containing an increase in solid component. ACC with grade 3 histology were associated with not only increased rates of treatment failure but also DNA aneuploidy (31). Their observations suggest that death typically occurs within three years from the time of diagnosis for an ACC with a solid pattern (31).

Microscopic perineural involvement has not been shown to negatively impact patient outcomes in numerous studies (8, 14, 17). However, any invasion of a named nerve by tumor cells results in a worse prognosis (14, 21, 22).

1.1.1.5   Biomarkers

Given the difficulties in diagnosing ACCs and the challenges and controversies surrounding their management, recent research has focused on the identification of molecular markers and/or hormonal receptor expression in these tumors.

1.1.1.5.1   C-kit (CD117)

The *c-kit* proto-oncogene encodes the transmembrane cell surface receptor known as c-kit. C-kit is a tyrosine kinase receptor involved in cell migration, proliferation, and differentiation (5). It can be found in both normal and abnormal tissues (28), and aberrant overexpression has been seen in certain tumors. The receptor can be detected by immunohistochemical staining for CD117 (32).

A limited number of researchers have studied the role of c-kit in salivary tissue (33). Andreadis et al. focused on the immunoexpression of c-kit in both benign and malignant salivary gland tumors (33). Of the 14 cases of ACC they examined, 80% showed c-kit expression. Furthermore, strong immunoreactivity was seen in all of the c-kit positive cases, with more than 50% of cells staining positive (33). Solid ACC or those with grade 3 growth patterns were noted to stain most intensely (33). Similarly, Jeng et al. found 80% (20/25) of ACC cases to be c-kit positive, and all ACC with a solid growth pattern expressed c-kit (34).

Earlier studies suggest that c-kit expression is limited to specific salivary gland neoplasms. Of the 79 salivary gland carcinomas studied by Jeng et al., c-kit positivity was seen in only 28 samples (34). The samples expressing c-kit belonged to one of three types of tumors, including ACC, lymphoepithelioma-like carcinoma and myoepithelial carcinoma (34). Penner et al. have also suggested that different salivary gland tumors

differentially express c-kit (35). Based on their observations they concluded that strong c-kit expression characterizes ACC, and it may aid in the differentiation between ACC and polymorphous low-grade adenocarcinoma (35).

Contrary to the works of Jeng et al. and Penner et al., others have shown that c-kit expression is not restricted to ACC or other malignant salivary gland neoplasms for that matter (33, 36). In the study by Chandan et al., c-kit expression was found in 100% (15/15) of pleomorphic adenoma samples and 100% (10/10) of ACC samples (36). Likewise, Andreadis et al. showed that c-kit is expressed in many different types of benign and malignant salivary gland tumors (33).

The ubiquitous expression of c-kit by different salivary gland neoplasms questions its usefulness as a marker to differentiate between tumor types (36). That being said, the near universal expression of c-kit by ACC specimens (33-36) suggests a potential role for tyrosine kinase inhibitors (35).

Imatinib mesylate is a tyrosine kinase inhibitor that targets ABL, platelet-derived growth factor receptor (PDGF-R) and c-kit (32). Its binding to the c-kit receptor inhibits cell proliferation and induces apoptosis (32). Given the overexpression of c-kit in ACC, Alcedo et al. explored the use of imatinib mesylate to target these tumors (32). In their study, they showed a reduction in tumor burden in two inoperable cases of ACC; one was in the context of recurrent disease, and the other was in the context of locally advanced disease (32). However, a multi-institutional phase II study was unable to duplicate the effectiveness of imatinib (37). All 16 patients, with c-kit positive ACC and unresectable or metastatic disease, enrolled in the study failed to show any objective response to imatinib therapy (37). Furthermore, Lin et al. noted rapid disease progression in 60%

(3/5) of patients with metastatic ACC, which were expressive for c-kit, when treated with imatinib over a 2 to 3 week period (38). The authors suggested that their case series might represent the first documented cases of imatinib-enhanced tumor growth (38).

2.1 Proteases

Proteases play a critical role in the functioning of all living cells (39), and approximately 2% of all human genes are devoted to encoding these proteins (40). The enzymatic activity of proteases is required for angiogenesis, neurogenesis, inflammation, apoptosis, hemostasis, immunity, fertilization, ovulation, wound repair, stem cell stimulation, and protein activation (39, 41). For proteases to perform these vital functions, they are ubiquitously present in all organelles of the body. Therefore, alterations in proteolytic function can have widespread pathological implications (41). Proteases have been implicated in a number of diseases including heart disease, cancer, neurodegenerative and autoimmune diseases (39, 41).

Proteases come in many shapes and sizes and vary in terms of specificity. Some proteases target only a specific peptide bond of a single protein while other indiscriminately target multiple substrates (41).

Proteases are grouped into classes based on their mechanism of catalysis (39). The six main classes are serine proteases, cysteine proteases, threonine proteases, aspartic proteases, glutamic proteases and metalloproteases (41). Proteases in each class can also be described as being exopeptidases, if they target the terminal peptide bond, or endopeptidases, if they cleave internal peptide bonds.

The MEROPS database, founded in 1996, classifies peptidases in a hierarchical fashion based on their degree of biochemical similarity (40). Proteins that share

homologous protein domains, also termed the peptidase unit, are grouped into families, and homologous families, which have similar three-dimensional structures and share a common ancestry, are grouped in clans (40, 41). For example, tissue kallikreins belong to the S1 family of serine proteases of the PA clan (Proteases of mixed nucleophiles, superfamily A) (42).

2.1.1   Serine Proteases

Serine proteases compromise over one-third of all known proteolytic enzymes (39, 43), and of all the protease classes, they have the most members (41). They are broadly disseminated throughout nature and can be found in all kingdoms of life (43). In total, there are 40 families and 13 clans of serine proteases (43). Of these 40 families, four are responsible for approximately 40% of all human proteolytic activity (43).

Serine proteases, like cysteine and threonine proteases, use an amino acid to guide the cleavage of peptide bonds. In the case of serine proteases, a serine residue at the active site of the protein acts as a nucleophile to facilitate enzymatic function (41). Under normal physiologic pH, serine residues are poor nucleophiles and must rely on the interaction with an acid and a base to improve their reactivity (44). Together, the acid-base-nucleophile or catalytic triad, as they are more commonly referred, perform covalent catalysis (44). During catalysis, the hydroxyl group of the serine nucleophile attacks the carbonyl group of the substrate yielding an acyl-enzyme intermediate (44). The generation of the acyl-enzyme intermediate ultimately lowers the activation energy for proteolysis and accelerates the chemical reaction (44).

The activity and function of serine proteases have widely been studied, particularly in humans. Commonly encountered proteases include trypsin, chymotrypsin,

thrombin, plasmin, elastase, and kallikrein (45). These enzymes are involved in digestion, coagulation, immunity, fertilization, development, neuromuscular functioning, apoptosis, blood pressure regulation, and malignancy (43, 45). Most of the serine peptidases involved in these biological processes are endopeptidases (43, 45)

PA clan proteases, as classified by the MEROPS database, play important roles in extracellular processes (43). Together, they constitute the largest family of serine proteases (43). All PA clan peptidases rely on the catalytic triad, Serine-Histidine-Aspartate (Ser/His/Asp), to perform catalysis (39). During a chemical reaction the serine residue acts as the nucleophile, the histidine residue acts as both an acid and base, and apartate helps orient the other residues and stabilize the charge buildup during the intermediate reaction (39).

3.1   Kallikreins

3.1.1   General Overview

The kallikrein family consists of 15 closely related serine proteases. The genes encoding these proteins are encoded in tandem on chromosome 19q13.3-13.4 and cover approximately 300 kb (46). Together, they constitute the largest protease gene cluster in the human genome (47).

The members of the KLK family share a similar homology in their DNA sequence (46). Between 35-85% is shared, with the classical *KLKs* (*KLK1-KLK3*) having the most closely related genomic organization (48). The remaining *KLK* genes (*KLK4-15*) display between 34.9%-46.2% sequence similarities with *KLK1* (49).

All kallikreins are formed by five coding exons of similar exon length and structure (46, 49). The start codon is located on exon 1, and the stop codon is located on

exon 5 (46, 49). The intron sequences (I, II, I, 0) are less conserved and more variable

(46). The gene lengths of these non-coding sequences vary between 4.3-10.5 kb (49).

3.1.2   Historical Overview

Kraut, Frey, and Werle discovered the first member of the kallikrein family in

1930 (50). Found in pancreatic extract, the novel peptidase was shown to have

vasoactive activity due to its ability to release bradykinin from kininogenase (42, 46).

This kinin-generating substance was named 'kallikreas', derived from the Greek word for

pancreas (46). Today, kallikreas is referred to as tissue kallikrein or KLK1 to

differentiate it from plasma kallikrein (46, 51).

Plasma kallikrein, also a serine protease with kininogenase activity, is synthesized

in the liver and circulates in the blood (46, 48). It is encoded by a single gene, *KLKB1*,

located on chromosome 4q34-35 and is unrelated to tissue kallikrein (46). Therefore, it is

not considered a member of the kallikrein family (42, 46).

Forty years after Werle's initial discovery, a second tissue kallikrein was

identified during the forensic study of semen (52). This newly identified peptidase was

termed prostate-specific antigen (PSA); it was later renamed KLK3 (52).

With advancements in technology and genomic sequencing, additional kallikreins

were discovered in the 1990s and the family continued its expansion into the millennium

(51, 52). Eventually, all 15 kallikrein genes were mapped to their corresponding loci.

In 2006, a comprehensive nomenclature system for the classification of

kallikreins was proposed and remains in use today (51). Under this system, kallikreins

without kininogenase activity are called kallikrein-related peptidases, and as a result, all

but tissue kallikrein (KLK1) were renamed (51). The names for KLK2-KLK15 changed

to kallikrein-related peptidase followed by the gene number (51). For example, KLK2 became kallikrein-related peptidase 2. The classification system is also used to distinguish between the gene and the protein. Each gene is referred to in italics as *KLK* followed by the gene number (e.g., *KLK2*) and each protein is written in standard font followed by the protein number (e.g., KLK2) (51).

3.1.3   Kallikreins as Proteases

Tissue kallikrein and kallikrein-related peptidases belong to the S1 family of serine endopeptidases (42), a subgroup of the PA clan, and display trypsin-like, chymotrypsin-like or dual activity (trypsin and chymotrypsin) (48). As with other serine proteases, the three residues forming the catalytic triad of KLKs are histidine, aspartic acid, and serine, and they are found at the active site of the enzyme (49).

Crystallographic analysis of six KLKs (KLK1, KLK3-7) has revealed a common archetypal tertiary fold structure (46). It consists of two adjacent, asymmetric beta-barrels and three interconnected loops (42, 48). This confirmation results in the formation of the active site. The protein is stabilized in this configuration by five disulfide bonds formed from 10 highly conserved cysteine residues (42). Externally, there are two exposed alpha-helices (46), and the structure is more variable resulting in different substrate specificity and regulatory functions for each KLK (49)

The catalytic triad residues, His57, Asp102 and Ser195, are encoded by the coding exons 2, 3, and 5, respectively (46). Their positioning is conserved in all 15 kallikreins (42, 48, 49). The serine residue acts as the nucleophile, the histidine residue as a proton donor and aspartic acid helps maintain the imidazole ring of histidine in a favorable orientation (48).

3.1.4   Kallikrein Activation

Kallikreins are translated as single-stranded intracellular pre-pro-enzymes (46, 49). Each pre-pro-KLK has a sequence length of 244-276 amino acids and is divided into three parts (49). The N-terminus domain consists of 16-30 amino acids, the pro-domain consists of 3-37 amino acids, and the core or catalytic domain consists of 227-252 amino acids (46, 50).

Removal of the signal peptide at the N-terminus domain results in the release of pro-KLKs into the secretory pathway (48, 50). Extracellularly, inactive pro-KLKs undergo further processing by either trypsin-like or metalloproteinase-mediated cleavage (53, 54). With the removal of the pro-domain, KLKs becomes active and undergo their characteristic conformational folding (49). Generation of active KLK through this mechanism is an important regulatory step in both normal physiology and disease (54).

3.1.5   Regulation of Kallikrein Activity

Tight control of kallikrein activity is critical for normal cell functioning. The generation of active KLKs from pro-KLKs is an irreversible step with the potential to cause a wide array of disorders if dysregulation occurs (49).

KLK regulation can occur at both the transcriptional and protein levels (50). At the transcriptional level, steroid hormones and promoter methylation play important roles (46).

The control of *KLK* expression by steroid hormones has been extensively studied (55). At least 14 hormone response elements have been identified in *KLK* genes (50). In a subset of tissues, steroid hormones including androgens, estrogens, progestins, mineralocorticoids, and glucocorticoids clearly regulate *KLK* expression (55). In the

prostate, *KLK2* and *KLK3* expression is responsive to androgen hormones (55). Upon androgen stimulation, androgen receptor binds to the androgen response element within the promoter region of *KLK2* or *KLK3,* which leads to the upregulation of KLK2 or KLK3, respectively. Breast tissue is another prominent site where androgens are involved in kallikrein expression. Similar to the prostate, *KLK3* expression appears to be androgen dependent (55). In fact, 98% of androgen receptor positive breast cancers express KLK3 (56).

Epigenetic factors have also been identified as important regulators of *KLK* expression (49). In certain malignancies, DNA methylation has been shown to impair tumor suppression (57, 58). One mechanism by which this occurs is through the aberrant methylation of CpG islands, which are GC rich regions commonly found in the promoter region of some genes. For example, the hypermethylation of CpG islands in acute lymphoblastic leukemia is involved in the downregulation of *KLK10* expression and is a poor prognostic factor (58). Similarly, in breast cancer, methylation causes a loss in KLK6 function leading to tumor progression and epithelial-mesenchymal transitions (EMTs) (57).

At the posttranscriptional level, KLKs can be regulated by small, non-coding microRNAs (miRNAs) (46). These miRNA have the ability to impair protein expression through translational inhibition or mRNA degradation (59). A single miRNA can target several KLKs, and therefore, it can exert an inhibitory effect on the expression of multiple KLK proteins simultaneously (59).

Once activated, the physicochemical microenvironment serves as the last control mechanism of KLK activity (49). Endogenous inhibitors, including irreversible serine

protease inhibitors (serpins), Kazal-type inhibitors and macroglobulins, prevent excessive proteolytic activity (60). Furthermore, Zn^{2+}, a single-metal-ion inhibitor, is important in attenuating KLK activity and preventing physiologic dysregulation (60). KLKs 2, 3, 4, 5, 7, 8, 12, and 14 are all attenuated by Zn^{2+} (60). The microenvironmental pH also regulates KLK activity (60). The histidine base (His57), at the enzymes active site, must be deprotonated to become catalytically active (61). In skin, elevated pH contributes to increased KLK levels (61). Lastly, kallikrein proteins may self-regulate their activity through internal autolytic inactivation (62). Experimental studies have shown that KLK6 undergoes self-internal cleavage as means to control enzymatic function (62)

Disruption of KLK regulation at any of these levels, whether it occurs during transcription, protease activation, or protease inactivation, can be pathologic. Anxiety, schizophrenia, neurodegeneration, skin-barrier dysfunction, respiratory disease, cancer and pathological inflammation have all been linked to aberrant control of kallikrein activity (42, 48).

3.1.6 Physiological Function of Kallikreins

Kallikreins are expressed in nearly all tissues throughout the human body (63). However, their relative abundances vary depending on the tissue type (42). For example, KLK2 and KLK3 are found in high concentrations only in the prostate (63). Others, such as KLK5-KLK8 and KLK13, are highly expressed in a restricted number of tissues, whereas KLK1, KLK4, KLK9-KLK12 and KLK14-KLK15 are ubiquitously expressed (42, 63). Certain kallikreins are co-expressed in specific tissues and participate with one another in proteolytic cascades (48, 55)

Glandular epithelia are primarily responsible for kallikrein secretion (52). These cells can be found in many organs including brain, skin, prostate, colon, breast and pancreas (52). As a result, KLKs are found in many bodily fluids such as breast milk, saliva, sweat, seminal fluid, cerebrospinal fluid and urine (63).

Kallikreins regulate tissue function and cell signaling through five distinct mechanisms (64). These mechanisms include the generation or inactivation of polypeptide agonists from precursor proteins, the release of membrane-anchored growth factors, the activation or inactivation of growth factor receptors or protease-activated receptors (PARs), and signaling via the extracellular matrix (ECM) or integrins (64). Kallikreins are also involved in important non-catalytic functions (46). Their interaction with other molecules and their chemotactic properties contribute to normal hemostasis (46).

KLKs have been implicated in physiologic pathways that regulate blood pressure, seminal liquefaction, kidney function, skin desquamation, tooth enamel formation, synaptic neural plasticity, and brain function (42). KLKs also play an important role in innate immunity (42).

3.1.7 The Role of Kallikreins in Malignancy

Aberrant *KLK* expression in malignant tissues suggests that kallikreins are involved in tumorgenesis (50). Research has shown KLKs contribute to the dissemination of cancer cells through their effects on cell migration and tissue invasion (49). It is well documented that KLKs have proteolytic activity against ECM proteins, cell membrane-bound receptors, cell adhesion proteins, and growth factors and signaling molecules (55, 65).

The degradation of ECM proteins, including fibronectin, laminin, vitronectin, and collagen I-IV, is important in the local dissemination and metastasis of tumor cells (49, 55). KLK7, which is overexpressed in pancreatic adenocarcinoma and other cancers, has been shown to cleave both fibronectin and E-cadherin in vitro (66, 67). The resultant disruption of the ECM architecture causes an increase in pancreatic cell invasion and a decrease in cell aggregation (67). Similarly, KLK13 has also been shown to degrade the ECM, and it has been suggested that it plays a role in the spread of certain malignancies, such as ovarian cancer, through the cleavage of collagen I-III, fibronectin, and laminin (68).

Kallikreins also exert pro-migratory effects through the modulation of EMT pathways (49). In head and neck squamous cell carcinoma EMT is enhanced by low KLK6 expression (69). In contrast, overexpression of KLK3 and KLK4 induces EMT in prostate cancer and facilitates the spread of tumor cells (70). Furthermore, KLKs can indirectly regulate the migration and invasion of tumors cells through modulation of the urokinase-type plasminogen activator (uPA)/plasmin system (49). KLK4 is involved in uPA activation through cleavage of the uPA receptor and the pro-enzyme of uPA (71).

KLKs may also influence carcinogenesis through the regulation of oxygen balance and inhibition of cell-mediated immunity (55, 72). For example, KLK3 is linked to the generation of reactive oxygen species independent of its proteolytic activity (73). KLK3 has also been shown to suppress the proliferation of T-lymphocytes in vitro (72).

Finally, it has been suggested that KLKs have pro-proliferative effects on cancer cells (49). Through the cleavage of insulin-like growth factor binding proteins (IGFBPs), the activity of IGF-1, an important growth factor in tumor proliferation, metastasis, and

angiogenesis, is disinhibited by kallikreins (74). KLK3 is involved in the dissociation of the IGF-1-IGF-binding-protein-3 (IGFBP3) complex, which leads to the proliferation of benign prostatic hyperplasia-derived stromal cells (75). In colon cancer, overexpression of KLK7 is associated with an increase in cell proliferation both in vitro and in vivo (76).

3.1.8   Kallikreins as Biomarkers

Biomarkers are widely used in medicine. Their primary function, regardless of whether they are used being used for screening, diagnosis or monitoring of disease, is to reduce mortality and increase length of life. One of the most studied cancer biomarkers is PSA, and its role in the detection of prostate cancer dates back over two decades.

The American Urological Association (AUA) and the American Cancer Society (ACS) first recommended annual PSA screening for men over the age of 50 in 1992 (77). This recommendation quickly became adopted into clinical practice, but as time has passed the role of PSA as a biomarker has become controversial (77, 78). In the Prostate, Lung, Colorectal, and Ovarian (PLCO) Trial, no reduction in mortality was seen in those screened with PSA (79).

Since the PLCO Trial, the rates of PSA screening has declined, and the U.S. Preventive Services Task Force has recommended against routine PSA-based screening for prostate cancer (77). In contrast, both AUA and ACS continue to recommend PSA testing. These recommendations are supported by two randomized control trials out of Europe where the number needed to screen to prevent one death was 333 in one study and 1410 in the other (80, 81).

If clinicians decide to offer PSA testing, they must be aware that screening leads to the overdiagnosis of prostate cancer due the test's low specificity (50, 82). In fact,

when PSA values between 2.5 and 4.0 µg/L are used as cutoffs, 80% of test results will be false positives (80).

The clinical accuracy of PSA can be improved by considering it in conjunction with KLK2 (50). When PSA values are greater than 4.0 µg/L, KLK2 is universally expressed and the ratio of KLK2 to free PSA has been shown to have improved specificity compared to PSA alone (83).

Kallikreins have also been studied as biomarkers in other malignancies. In ovarian cancer, the overexpression of KLK4 and KLK5 is associated with a poor prognosis (84). In contrast, KLK5 is a favorable prognostic factor in testicular cancer (85). Patient survival outcomes in non-small cell lung carcinoma, laryngeal cancer, and low rectal carcinoma have also been linked to kallikrein expression, specifically KLK11 (86-88). In renal cell carcinoma, high KLK6 expression is correlated with unfavorable outcomes (89). KLK6 is also associated with a poor prognosis in gastric cancer patient (90), and it is differentially expressed in uterine papillary serous carcinoma (91).

Chapter 2

2   Hypothesis and Rationale

2.1   Hypothesis

In this study, we hypothesize that kallikrein expression in ACCs will differ from that of normal salivary gland tissues (NSGTs). We further hypothesize that differential and aberrant expression of certain *KLKs* in ACC may serve as useful biomarkers in the management of these tumors.

2.2   Rationale

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. This not only complicates management but worsens patient morbidity and mortality.

Our ability to detect ACCs early and treat them with minimally invasive therapy is currently lacking. The future discovery of predictive and prognostic tests may facilitate early detection and identification of aggressive tumors.

Kallikreins have been implicated in a wide variety of signaling and regulatory roles, and their dysregulation has been associated with tumorgenesis. 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. However, 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.

2.3   Aims and Objectives

Specific Aim 1: To analyze the expression of *KLKs* in ACCs and NSGTs using real-time quantitative polymerase chain reaction (RT-qPCR).

Chapter 3

3   Materials and Methods

3.1   Tissue Specimens

Formalin-fixed, paraffin-embedded tissue specimens (FFPE) for both ACC and NSGT 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 the graduate student (ZK) and a senior oral pathologist to confirm the diagnoses. Only specimens where the tissue of interest (e.g. either ACC or NSGT) encompassed the majority of the sample were selected. Any normal samples containing areas of inflammation were excluded. In total, 25 ACCs and 15 NSGTs were deemed suitable for the study. Tables 3.1 and 3.2 list patient demographic information, including age, sex, and location of the lesion, for the ACC and NSGT specimens. Demographic information was unavailable for two of the ACC samples.

Table 3.1: Demographic Information for the ACC Specimens

Age	Sex	Location
61	Male	Oral mucosa
55	Male	Left hard palate
79	Female	Right palate
55	Male	Upper lip vestibule
64	Female	Right buccal mucosa
69	Female	Left soft palate
87	Female	Left buccal mucosa
63	Female	Left hard palate
53	Male	Hard palate
84	Female	Right maxillary vestibule
77	Female	Right palate
51	Male	Right upper lip
86	Female	Left maxillary tuberosity
64	Female	Left parotid gland
12	Male	Left submandibular gland
52	Female	Submandibular gland
53	Female	Left submandibular gland
67	Male	Right submandibular gland
77	Male	Left maxillary sinus
84	Female	Right parotid gland
77	Female	Right buccal mucosa
60	Female	Maxillary buccal vestibule
75	Male	Right neck
		Not available
		Not available

Table 3.2: Demographic Information for the NSGT Specimens

Age	Sex	Location
75	Female	Left gingiva
38	Female	Left lower lip mucosa
53	Female	Lower lip
20	Male	Right lower lip
24	Male	Right buccal mucosa
68	Female	Left lower lip
24	Female	Floor of mouth
20	Female	Lower lip
47	Female	Lower lip
42	Female	Right lower lip
18	Male	Left lower lip
68	Male	Uvula
55	Male	Right lower lip
33	Female	Lower lip
58	Female	Left hard palate

3.2   Sectioning of FFPE Specimens

The H&E slides for both ACC and NSGT were first examined to identify the areas on the slides consistent with the histopathological diagnosis. These areas were circled with a marker and then transcribed onto the corresponding areas on the FFPE blocks. A scalpel was used to score the marks onto the paraffin creating an outline around the section of interest.

With the use of a microtome (Microm HM 325, GMI Inc., Ramsey, MN, USA), 10 μm serial sections were cut from each FFPE block. Tissue forceps were then used to carefully separate each specimen from the surrounding areas along the previously placed scalpel lines. Lastly, the specimens were placed into 1.5 mL collection tubes.

3.3 **Deparaffinization of FFPE Specimens**

Removal of the paraffin from the FFPE specimens was as follows:

1. Xylene was added to the collection tubes containing the FFPE specimens.

2. The collection tubes were briefly shaken with a vortex mixer (3 times for 4 seconds) and allowed to incubate for 2 minutes.

3. The collection tubes were spun at 12,000 x g for 2 minutes.

4. The separated supernatant was discarded.

5. The above four steps were repeated.

6. Absolute alcohol was mixed with the residual specimens.

7. The collection tubes were spun at 12,000 x g for 2 minutes.

8. 70% ethanol was added, and the collection tubes were vortexed (3 times for 4 seconds) and centrifuged once again for 2 minutes at 12,000 x g.

9. The separated supernatant was discarded.

10. The collection tubes were spun one last time for 20 seconds, and all residual fluid was completely removed.

11. The tissue pellet in each tube was dried in a heating block for 15 minutes at 55°C.

3.4 **RNA Isolation from Deparaffinized Specimens**

RNA was isolated from the deparaffinized specimens using the High Pure FFPE RNA Micro Kit (Roche Applied Sciences, Mannheim, Germany, Catalogue number: 04823125001). The RNA isolation protocol was as follows:

1. Tissue Lysis Buffer and 10% sodium dodecyl sulfate was added to the deparaffinized specimens in each collection tube.

2. The collection tubes were shaken in the vortex mixer (3 times for 4 seconds) and

centrifuged.

3. Prepared Proteinase K solution was added, and each collection tube was vortexed (3 times for 4 seconds) and centrifuged.

4. The specimens were incubated for 3 hours at 55°C.

5. Binding Buffer and absolute ethanol were added, and each collection tube was shaken in the vortex mixer (3 times for 4 seconds) then centrifuged.

6. The cell lysate from each collection tube was placed into the upper reservoir of a High Pure Filter Tube with a pipet.

7. Each Filter tube, placed inside the corresponding collection tube, was centrifuged at 8,000 x g for 30 seconds, and the flow-through was discarded.

8. The filter was dried by centrifuging at maximum speed for 1 minute.

9. Prepared DNase solution was added, and each tube assembly was allowed to incubate for 15 minutes at 25°C.

10. Prepared Wash Buffer I was added, and tube assemblies were centrifuged at 8,000 x g for 15 seconds. The flow-through was discarded.

11. Prepared Wash Buffer II was added and tube assemblies were centrifuged at 8,000 x g for 15 seconds. The flow-through was discarded.

12. Step 11 was repeated except this time each collection tube was discarded.

13. The Filter Tubes were placed inside new collection tubes then centrifuged at maximum speed for 2 minutes to once again dry the filters. The collection tubes were then discarded.

14. Each Filter Tube was placed into new collection tubes, and Elution buffer was added.

15. The tube assemblies were incubated at 25°C for 1 minute then centrifuged at 8,000 x g for 1 minute.

16. The eluate was reloaded, and step 15 was repeated.

17. Each collection tube now contained isolated RNA.

3.5   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). The reaction protocol was as follows:

1. 405 μL of Nuclease-free water, 180 μL of 5x iScript Reaction Mix and 45 μL of iScript Reverse Transcriptase were mixed in a 1.5 mL collection tube.

2. 16 μL of the mixed reagents was then mixed with 4 μL of RNA, which had been previously isolated from the FFPE specimens.

3. The reaction was completed in a CFX Connect thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following protocol: priming for 5 minutes at 25°C, reverse transcription for 20 minutes at 46°C and reverse transcription inactivation for 1 minute at 95°C.

3.6   SYBR Green RT-qPCR of Synthesized cDNA

RT-qPCR 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 3.3.

Previously synthesized cDNA specimens (20 µL) were mixed with 225 µL of RT^2 SYBR Green qPCR Mastermix (Qiagen, Toronto, ON, Canada) and 205 µL of water. 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 used is shown in Figure 3.1 and was as follows: denaturation for 10 minutes at 95°C, 75 cycles of amplification for 15 seconds at 95°C and 1 minute at 60°C.

The specificity of PCR amplification was verified by post RT-qPCR melting curve analysis and agarose gel electrophoresis.

Table 3.3: Primer Assays from Qiagen used for RT-qPCR

Primer Assay	Catalog Number
β-actin	QT01680476
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

Figure 3.1: RT-qPCR Protocol for cDNA Amplification with *KLK* Primers
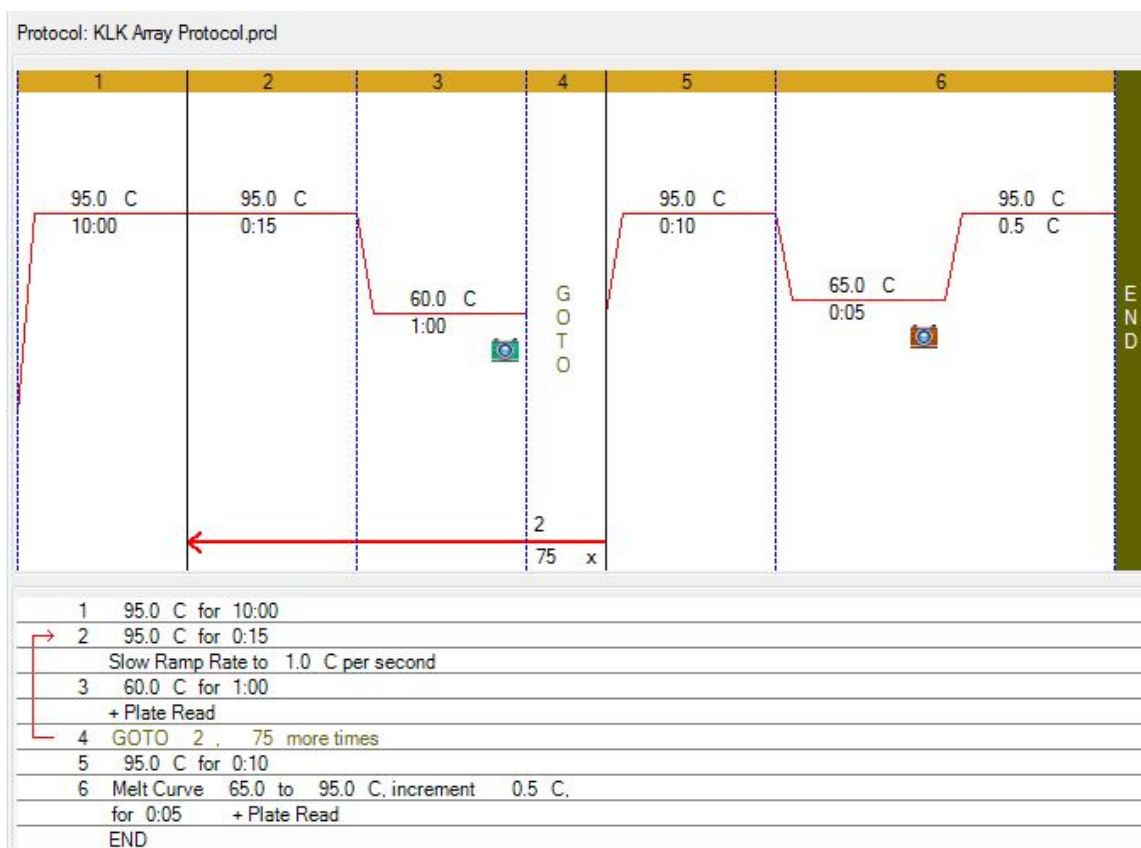
3.7   Melting Curve Analysis of PCR Products

The generated melting curves were analyzed for each PCR product. Table 3.4 lists the known melting temperatures (T_m) for *KLK1-KLK15* and *β-actin*. These values, which were provided by the manufacturer, 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 3.4: Melting Temperature for *β-actin* and *KLK1-15* Primers

Primer	T_m Values (± 0.5°C)
β-actin	85.5
KLK1	82.5
KLK2	82.5
KLK3	84.5
KLK4	83.5
KLK5	79.5
KLK6	81.5
KLK7	84.5
KLK8	82.5
KLK9	85.5
KLK10	82.0
KLK11	80.5
KLK12	81.0
KLK13	85.0
KLK14	85.0
KLK15	84.0

3.8   Agarose Gel Electrophoresis of PCR Products

The products of PCR were visualized using agarose gel electrophoresis through the following steps:

1. A running buffer was prepared by adding 20 ml of TAE [50X] (2M Tris-acetate, 0.05M EDTA, pH 8.3) to 1000 ml of distilled water.

2. 100 ml of the running buffer was added to an Erlenmeyer flask containing 2 g of Agarose PCR Plus.

3. The flask was repeatedly swirled and heated until the mixture was clear.

4. Ethidium bromide (final concentration 0.07 μg/ml) was added to the 2% agarose gel.

5. The gel was brought to a boil then allowed to cool to approximately 60°C.

6. The gel was poured into a casting tray containing a single comb, which created 20 wells.

7. The gel was allowed to cool and set before removing the comb.

8. 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.

9. The chamber was filled with the running buffer until the gel was completely covered.

10. The first well was loaded with 12 μl of TrackIT 100 bp DNA Ladder.

11. The next 15 wells were loaded with 12 μl of amplified *KLK1* through *KLK15* PCR products, which each contained 1 μl of DNA Gel Loading Dye [6X].

12. The chamber was connected to the power source, and an electric current was applied.

13. After adequate migration, the gel was removed and placed on an ultraviolet viewing box.

14. Digital photographs were taken of the gel.

3.9   Statistical Analysis

Relative quantification using the ΔCq method was used to analyze the RT-qPCR data. For each specimen, the quantification value (Cq) of individual *KLKs* was normalized to the Cq value of *β-actin*, a housekeeping gene. The Cq values were transformed into ΔCq values using Bio-Rad CFX Manager 3.0 (Bio-Rad Laboratories, Hercules, CA, USA).

The ΔCq values were calculated using the formula $\Delta Cq = 2^{Cq(\beta\text{-}actin) - Cq(KLK)}$. The specimen with the lowest Cq value for a particular *KLK* had its ΔCq value set to 1. It was then used as a calibrator to convert the ΔCq values of the remaining specimens to relative quantities.

The calculated relative quantities for each *KLK* were compared between ACC and NSGT to look for differences in expression. The non-parametric Mann-Whitney U Test was used for the statistical analyses. The level of significance was set at 0.05 ($p<0.05$). The analyses were performed using GraphPad software (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, California, USA).

Chapter 4

4   Results

4.1   Specimens Expressing *KLK* cDNA in ACC and NSGT

The expression of kallikreins (*KLK1-KLK15*) was examined in ACC and NSGT using RT-qPCR. As shown in Table 4.5, all members of the kallikrein family were expressed in both ACC and NSGT. Three of the ACC specimens and two of the NSGT specimens were excluded from the study following the review of their melting curves due to aberrant *β-actin* amplification, which was reflected in discrepancies between the observed melt peak T_m values and that expected in Table 3.4.

KLK9 was the kallikrein least likely to be detected in NSGT and it was found in only 38.5% of samples. In ACC, *KLK9* and *KLK13* were both expressed in a smaller number of specimens compared to the other kallikreins and 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.

Table 4.5: Percentage of ACC and NSGT Expressing *KLK1-KLK15*

Gene	ACC	NSGT
KLK1	73.0	84.6
KLK2	82.0	69.2
KLK3	68.0	84.6
KLK4	64.0	61.5
KLK5	82.0	84.6
KLK6	68.0	76.9
KLK7	64.0	69.2
KLK8	82.0	76.9
KLK9	45.0	38.5
KLK10	73.0	69.2
KLK11	68.0	84.6
KLK12	86.0	100
KLK13	41.0	61.5
KLK14	91.0	84.6
KLK15	77.3	69.2

4.2   **Comparison of *KLK* cDNA Expression Between ACC and NSGT**

Differences in kallikrein cDNA expression in ACC and NSGT were compared using the ΔCq method.

Figures 4.2-4.5 depict the specific ΔCq values from each specimen for each *KLK*. The data is arranged in a scatter plot with a horizontal line representing the median ΔCq value.

KLK1, *KLK8*, *KLK11*, and *KLK4* were all found to have significantly lower ΔCq values in ACC compared to NSGT. In contrast, no statistical difference in ΔCq values were seen for *KLK2-KLK7, KLK9, KLK10, KLK12, KLK13* and *KLK15*. The calculated two-tailed p-values are presented in Table 4.6.

For *KLK1, KLK8, KLK11,* and *KLK14*, median and quartile ΔCq values for both tissue types are presented in Table 4.7 and Figure 4.6. 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.

Figure 4.2: Scatter Plots of ΔCq Values for *KLK1-4* in ACC and NSGT. Statistical Comparison using Mann-Whitney U Test. *p≤0.05; **p≤0.01
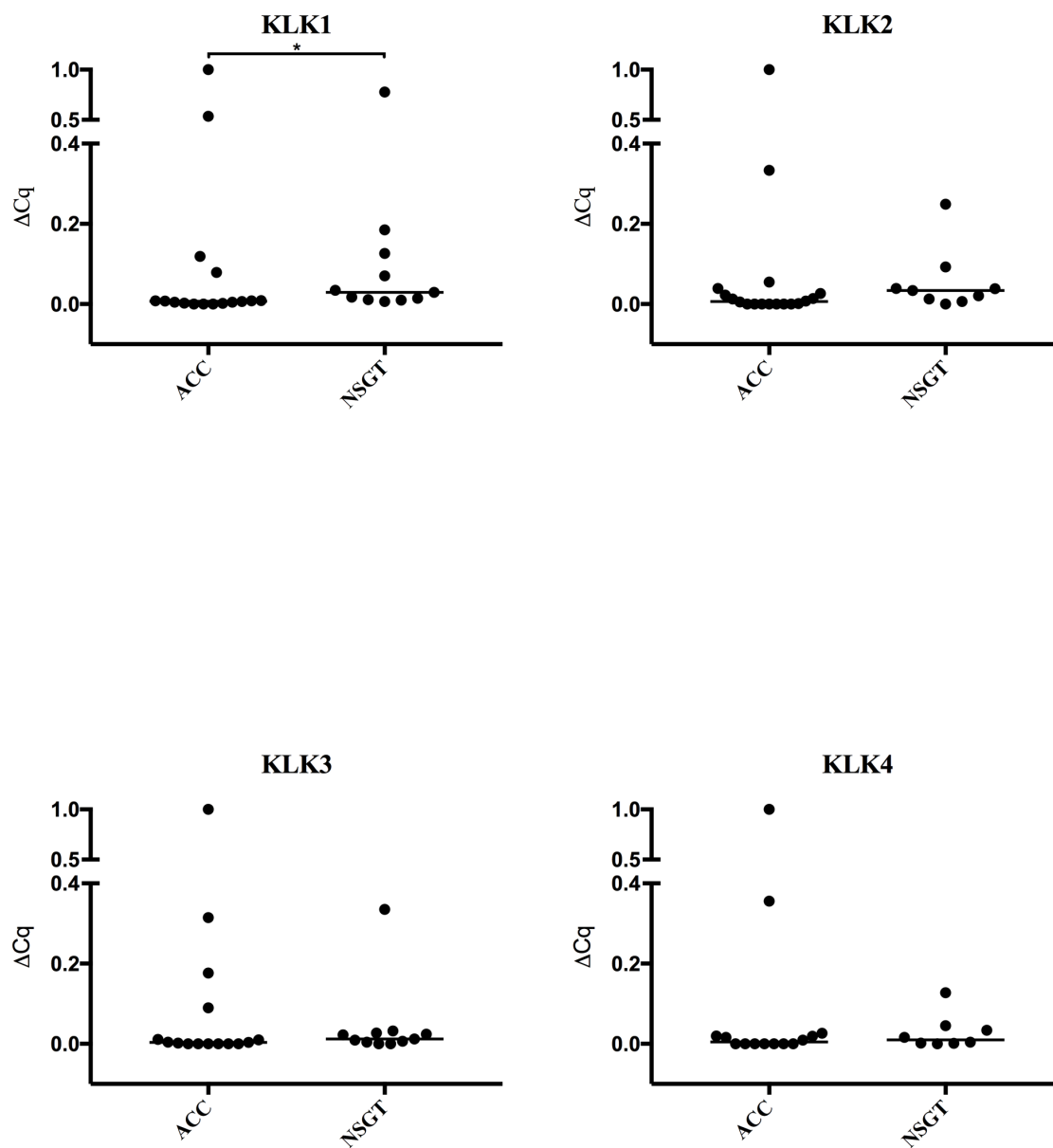
Figure 4.3: Scatter Plots of ΔCq Values for *KLK5-8* in ACC and NSGT. Statistical Comparison using Mann-Whitney U Test. *p≤0.05; **p≤0.01
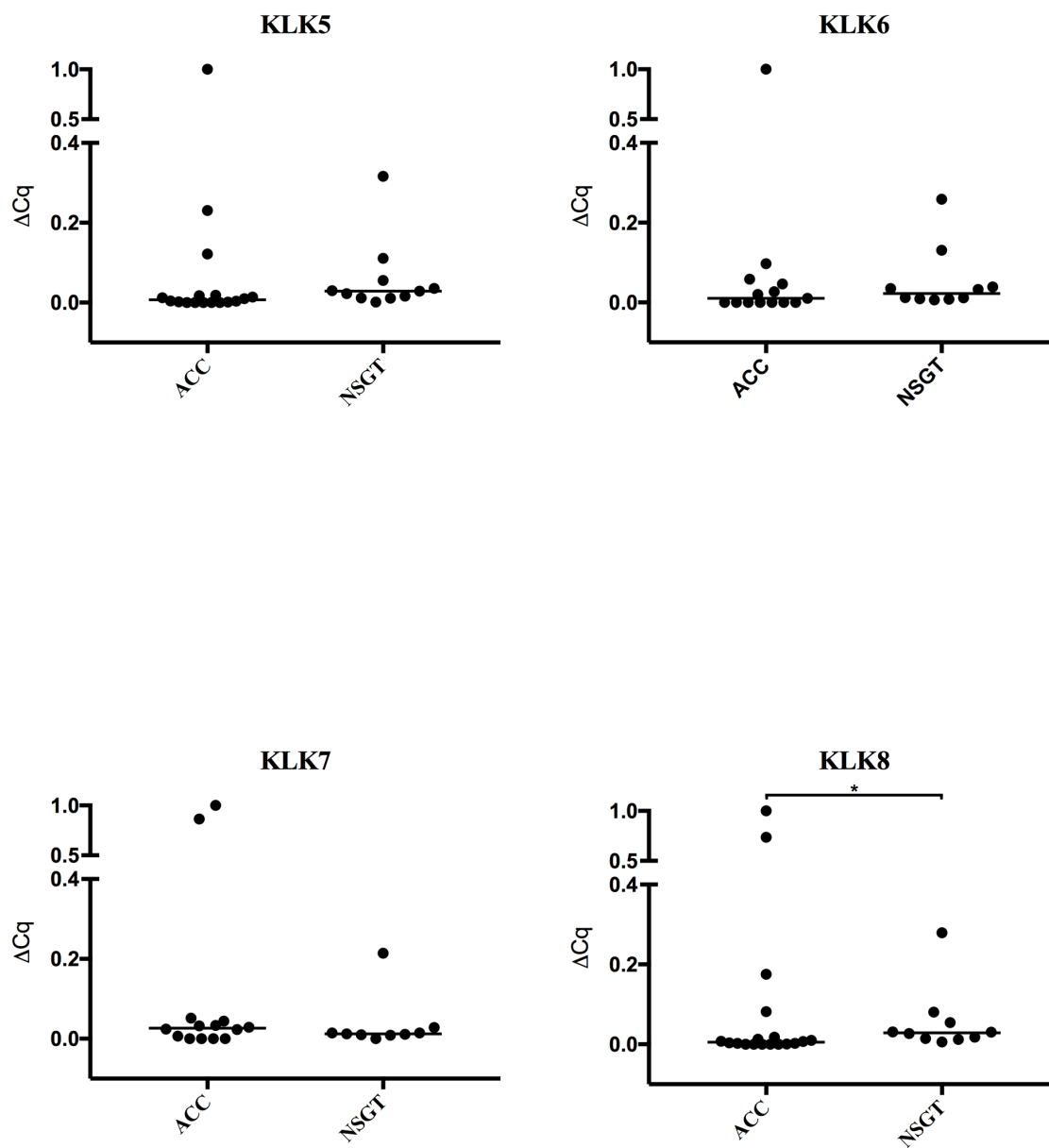
Figure 4.4: Scatter Plots of ΔCq Values for *KLK9-12* in ACC and NSGT. Statistical Comparison using Mann-Whitney U Test. *p≤0.05; **p≤0.01
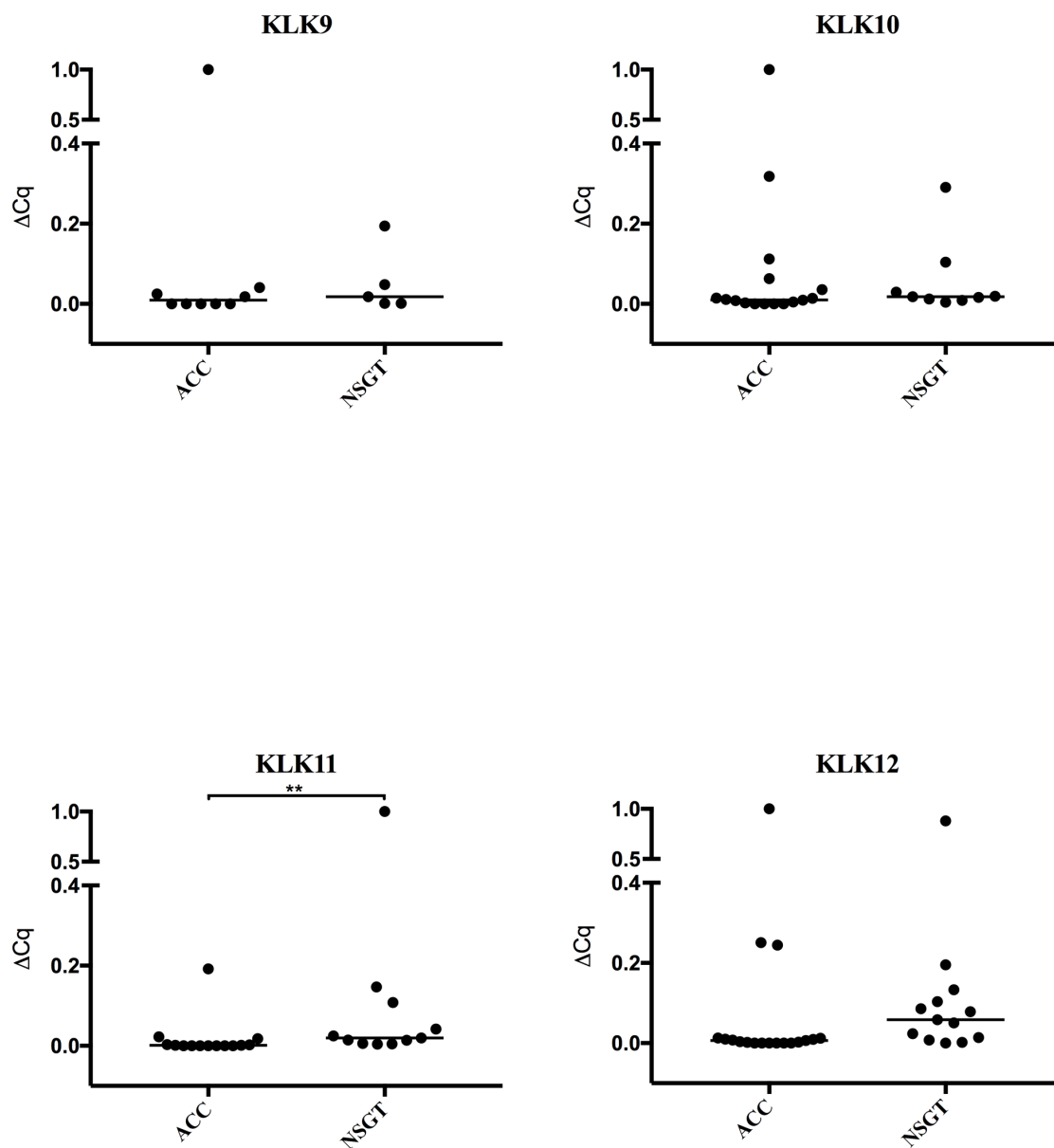
Figure 4.5: Scatter Plots of ΔCq Values for *KLK13-15* in ACC and NSGT. Statistical Comparison using Mann-Whitney U Test. *p≤0.05; **p≤0.01
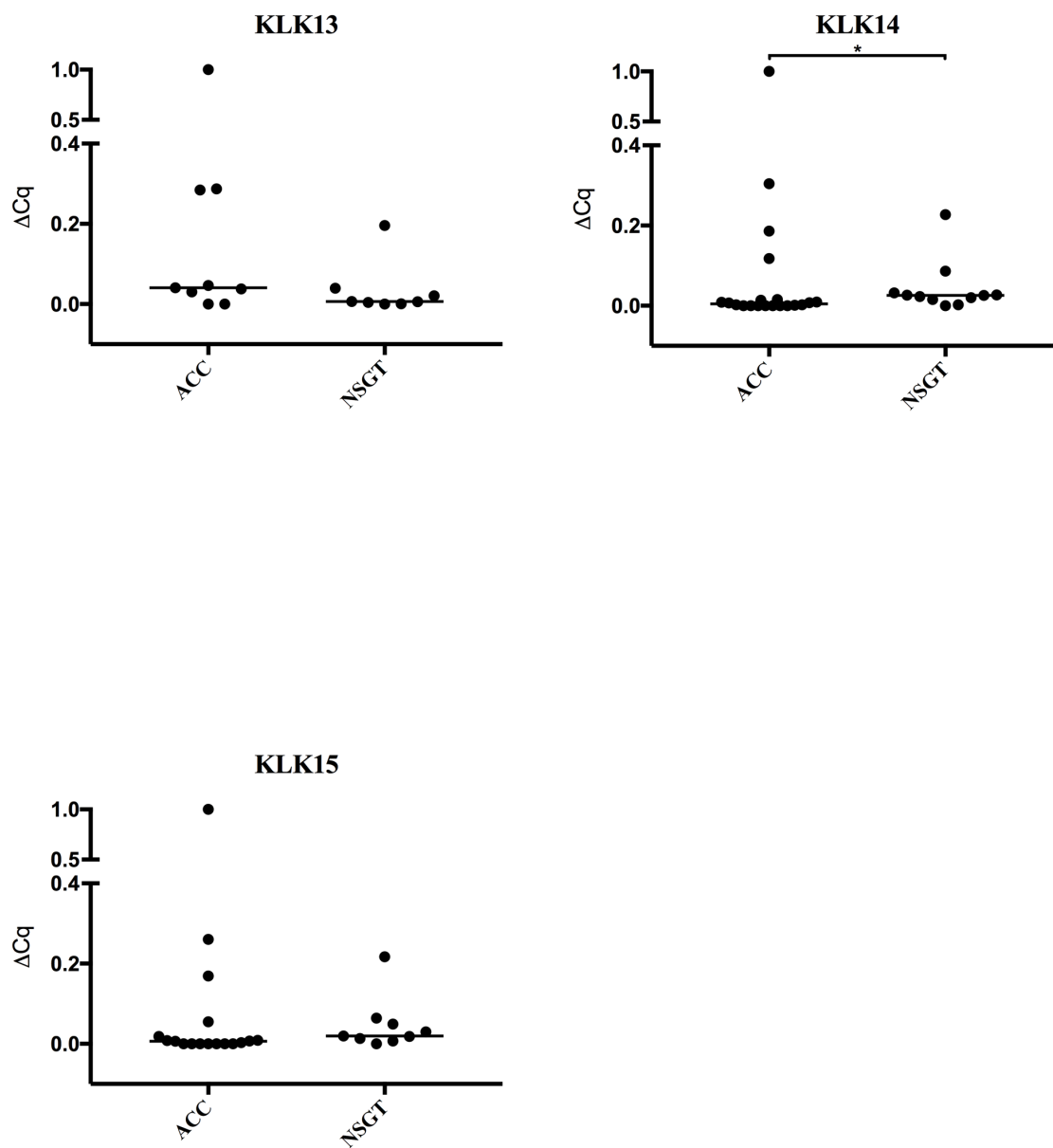
Table 4.6: Calculated Two-Tailed P-values from the Statistical Comparison of ΔCq Values Between ACC and NSGT using Mann-Whitney U Test

Gene	Two-tailed P-value
KLK1	0.0198
KLK2	0.1061
KLK3	0.2645
KLK4	0.4525
KLK5	0.0922
KLK6	0.3383
KLK7	0.5571
KLK8	0.0400
KLK9	0.5135
KLK10	0.2756
KLK11	0.0064
KLK12	0.0655
KLK13	0.1996
KLK14	0.0440
KLK15	0.1382

Table 4.7: Comparison of Median and Quartile ΔCq Values in ACC and NSGT 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
	NSGT	0.02903	0.01288	0.09830
KLK8	ACC	0.00543	0.00013	0.01682
	NSGT	0.02862	0.01543	0.04847
KLK11	ACC	0.00146	0.00000	0.01030
	NSGT	0.01959	0.00978	0.07486
KLK14	ACC	0.00467	0.00014	0.01420
	NSGT	0.02583	0.01790	0.02944

Figure 4.6: Box and Whisker Plots of ΔCq Values for *KLK1*, *KLK8*, *KLK11*, and *KLK14* in ACC and NSGT. Statistical Comparison using Mann-Whitney U Test. *p≤0.05; **p≤0.01
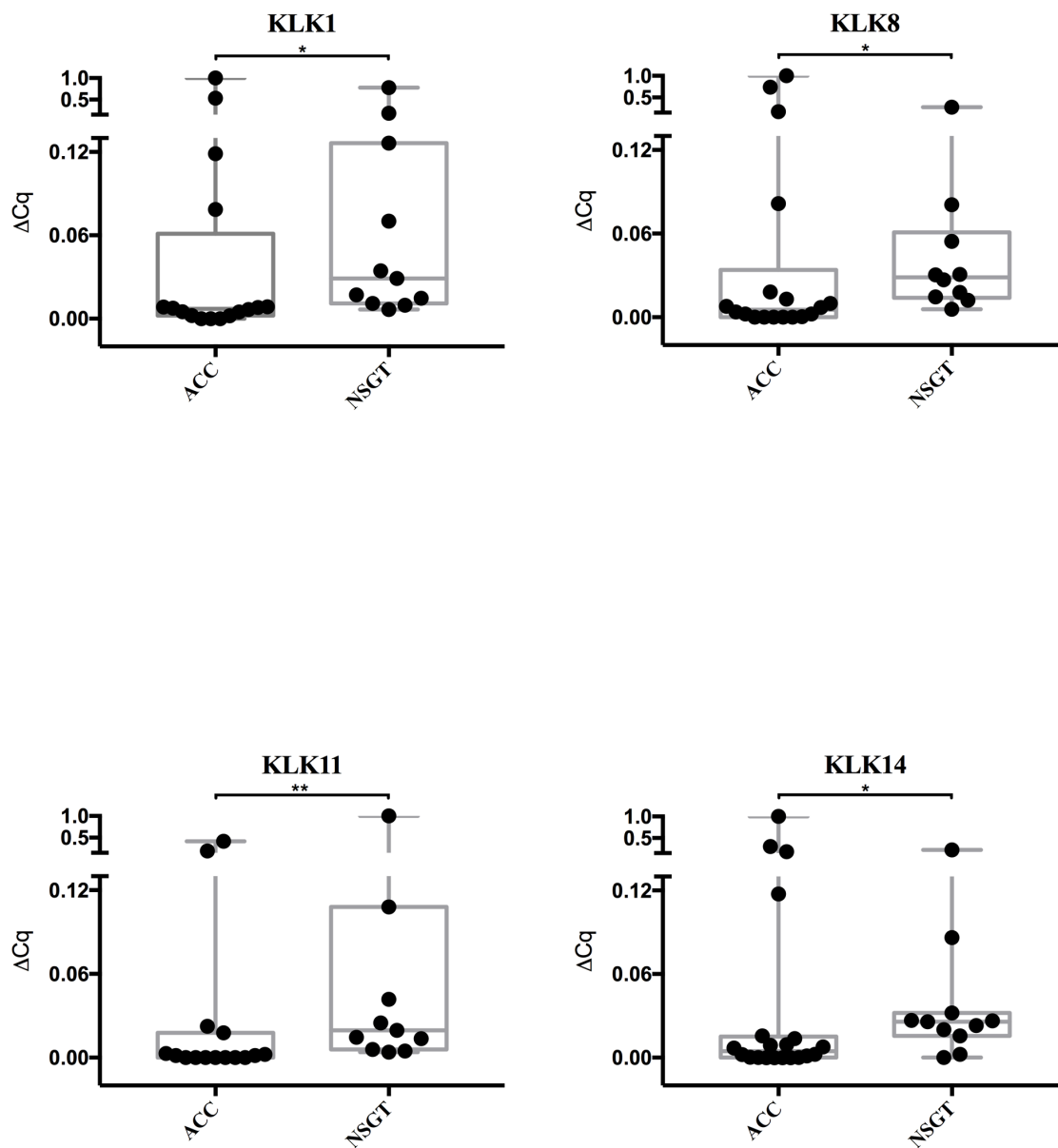
4.3 **Verification of PCR Products on Agarose Gel Electrophoresis**

PCR products were analyzed by agarose gel electrophoresis for the identification of *KLK1-15* amplicons.

The four gels shown in Figure 4.7 confirm good primer specificity. In Figure 4.8, 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 reference to the Table 4.8, confirms the presence of *KLK1-15* and ultimately the methodology of cDNA synthesis from RNA isolated from FFPE specimens.

Figure 4.7: Agarose Gel Electrophoresis of PCR Products *KLK1-15* in ACC (A-C) and NSGT (D)
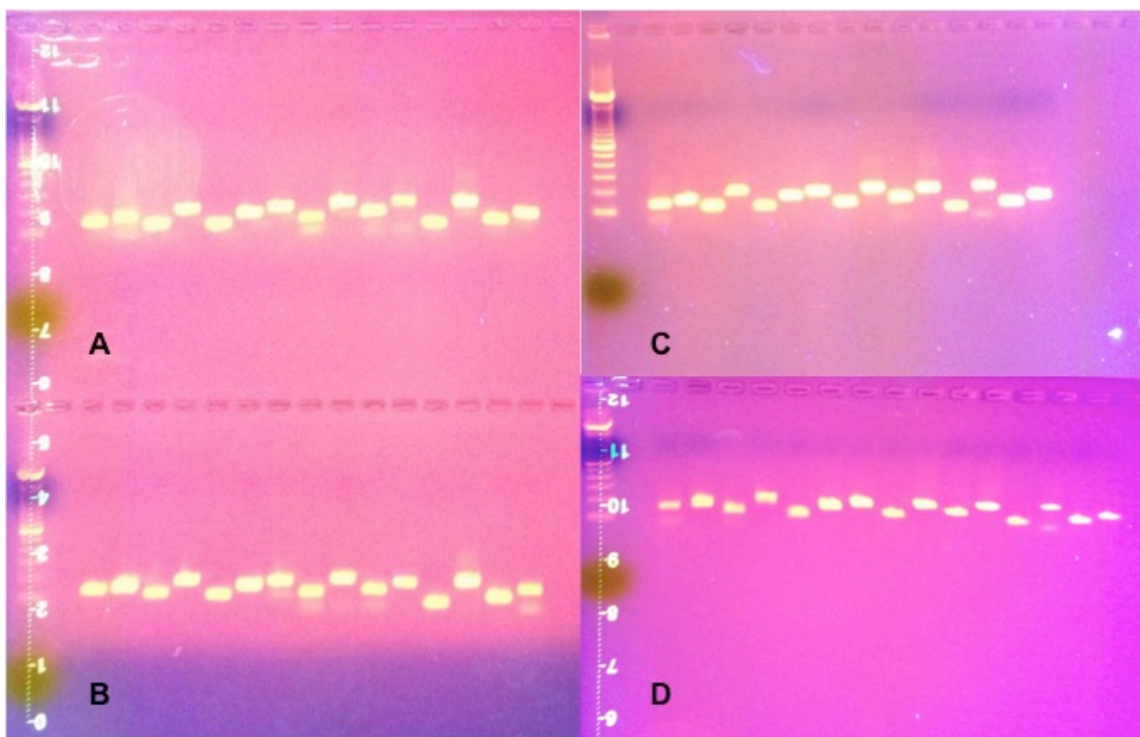
Figure 4.8: Agarose Gel Electrophoresis of PCR Products from ACC Showing Band Separation of *KLK1-15* Amplicons in Reference to TrackIT 100 bp DNA Ladder

Table 4.8: Number of Base Pairs for *KLK* Amplicons

KLK Amplicon	Number of Base Pairs
KLK1	118
KLK2	134
KLK3	101
KLK4	172
KLK5	96
KLK6	146
KLK7	171
KLK8	114
KLK9	184
KLK10	134
KLK11	183
KLK12	81
KLK13	185
KLK14	104
KLK15	135

Chapter 5

5   Discussion

The kallikrein family constitutes the largest cluster of proteases within the human genome, and all 15 *KLK* genes are encoded in tandem on chromosome 19q13.3-13.4 (47). All KLKs share similarities in their DNA sequences and tertiary structures suggesting conserved function amongst the encoded proteins (42, 46). They have been reported to play important roles in physiological pathways, such as blood pressure control, semen liquefaction, skin desquamation and innate immunity (42). Kallikreins have also been implicated in pathologic processes. Studies have shown that KLKs influence tumorigenesis through their effects on EMT, disruption of normal oxygen balance, degradation of the ECM, and involvement in tumor cell proliferation (49, 55).

KLK3 has been the most widely studied kallikrein, and it exemplifies the families' potential role as biomarkers (92). Furthermore, the discovery of extra-prostatic expression of KLK3 in salivary tissues, using in situ hybridization and RT-PCR, suggests that KLKs may be involved in salivary tumors (93).

Shaw et al. identified the presence of all members of the kallikrein family in salivary glands at the time of post-mortem autopsy using semiquantitative RT-PCR of human tissue extracts (63). Previous research in our laboratory has demonstrated either increased or decreased immunohistochemical expression of KLK5, KLK6, KLK8, KLK10, KLK13 and KLK14 in salivary tumors compared to NSGTs (94-99). Based on these findings, Darling et al. has stated that kallikreins may serve as valuable biomarkers for the diagnosis and monitoring of salivary gland carcinomas (95, 97).

In this study, we have reconfirmed that all human kallikreins are expressed in NSGT using RT-qPCR. However, to our knowledge we are the first group to explicitly

exclude samples with any evidence of inflammation. We feel this is important given that previous researchers have implicated the involvement of kallikreins in inflammatory processes, and histological inflammation can affect KLK expression levels (42, 48). For example, elevated PSA levels have been observed in patients with benign conditions such as prostatitis or benign prostatic hyperplasia (100). Okada et al. have shown that inflammation within biopsy specimens correlates with elevated total PSA levels (101). Furthermore, subclinical inflammation seems to not only increase total PSA levels but also decreases free PSA, in much the same manner as prostate cancer (100). This makes interruption of PSA values in the gray zone (4-10 ng/ml) difficult and lowers the test's specificity. Based on this information it seemed only appropriate to first compare non-inflamed NSGT to ACC specimens in the initial evaluation of the utility of KLKs as biomarkers in salivary gland tumors.

As previously stated, the presence of KLKs has been studied at both the protein and nucleic acid level in NSGT and salivary gland tumors. However, this is the first time kallikrein expression in NSGT and ACC has been measured using RT-qPCR.

In this study, ACC has been shown to express significantly lower levels of *KLK1, KLK8, KLK11* and *KLK14* compared to NSGT. Of these kallikreins, KLK8 and KLK14 have previously been studied in ACC tissues using immunohistochemical expression (96, 98).

KLK8 is a favorable prognostic marker in ovarian and non-small cell lung cancer (102-104). It is known to suppress cancer metastasis (103), and it is indicative of low-grade disease and improved overall survival (102, 103). Experimental studies have shown that overexpression of *KLK8* suppresses the invasiveness of cancer cell lines

(103). Furthermore, KLK8 retards cancer cell motility by inhibiting integrin signaling and actin polymerization through the cleavage of fibronectin (103). Sher et al., using microarray analysis, found that the overexpression of *KLK8* downregulates VEGF signaling, a key inducer of angiogenesis and cancer development (103).   In breast cancer, *KLK8* expression has been shown to be downregulated using RT-PCR (105).

In our study, we have demonstrated decreased expression of *KLK8* in ACC compared to NSGT. Previous work in our laboratory showed that malignant salivary gland tumors express significantly higher levels of KLK8 than normal salivary glands (96). However, the difference in expression was largely due to increases in expression in mucoepidermoid carcinoma and adenocarcinoma not otherwise specified (96).   As for ACC, no significant difference in immunoreactive levels was observed compared to normal controls (96).

Growing evidence suggests that both kallikrein gene and protein expression are commonly upregulated or downregulated in coordination (106)   Therefore, based on our observation of decreased *KLK8* expression one would expect the protein levels of KLK8 to be decreased in ACC, but instead they appeared to be unchanged. One possible explanation for this finding is that the immunoperoxidase staining technique used by Darling et al. was not sensitive enough to detect subtle differences in expression between ACC and the controls.

Unfortunately, the clinical outcomes of the patients included in this study are unavailable. Thus, we can only speculate about the potential role of kallikrein 8 in ACC development. It is possible that *KLK8* downregulation eliminates its ability to suppress tumor cell invasion and may provide an explanation for ACC's propensity for perineural

spread. Also, *KLK8* downregulation may facilitate hematogenous spread through VEGF upregulation.

KLK14 expression is under steroid hormone regulation, and it has a restricted expression pattern (107). It is found predominantly in the central nervous system and endocrine-related tissue (107). In prostate tissue, *KLK14* upregulation is seen in cancerous tissue compared to non-cancerous tissue independent of PSA values and higher expression levels correlate with advanced and aggressive disease (108). With respect to breast cancer, the role of KLK14 as a marker is less clear. Yousef et al. reported a loss of *KLK14* expression in 21 of 25 breast cancer samples analyzed using RT-PCR (109). In contrast, Papachristopoulou et al. observed higher *KLK14* expression in malignant breast tumors compared to benign tumors (110). In addition, *KLK14* overexpression has been associated with increased breast tumor size and stage (110, 111) and increased KLK14 protein levels have been linked to positive nodal status (111).

Studies have shown that KLK14 can activate PAR2 (112, 113). PAR2 belongs to a family of four ubiquitous G-protein coupled receptors (PAR1-4), known as protease-activated receptors (PARs) (106). PARs play a diverse role in physiologic processes such as hemostasis, inflammation, and wound healing (114, 115). PAR signaling has also been linked to carcinogenesis and has been identified in numerous tumors (115). Based on an in vitro study, it has been suggested that aberrant KLK14 expression activates PAR2 leading to colon cancer proliferation (116). It would be interesting to determine if PAR2 and KLK14 are co-expressed in ACC. It has previously been shown PAR2 is not only expressed in normal salivary glands, but it is involved in the control of normal exocrine function (117). The presence of PAR2 in salivary gland neoplasms has

not been confirmed, and it is possible that KLK14 may mediate their development through PAR2 signaling.

Previously published results from our laboratory found KLK14 to be expressed in normal salivary glands, pleomorphic adenomas, ACCs and mucoepidermoid carcinomas (98). In ACC, the immunoreactivity of KLK14 was increased compared to normal control tissue but did not reach statistical significance (98). In our study, we have found downregulation of *KLK14* at the DNA level.

Yousef et al. have previously reported discrepancies between gene and protein expression for many kallikreins (118). They went on to show that miRNAs play a role in KLK protein expression through post-transcriptional control mechanisms (59). A single miRNA was found to have the ability to target multiple kallikreins and simultaneously turn off their protein expression (59). Our laboratory findings appear unique in that protein levels seem upregulated and gene expression is downregulated for *KLK14* in ACC. Additionally, KLK13 expression has been found to be significantly increased in ACC compared to normal control (97), yet no difference is seen between in *KLK13* levels.

In some rare instances, protein translation has been enhanced by miRNAs in non-kallikrein studies (119-121). Vasudevan et al. have shown that miRNAs can either enhance or repress translation depending on differences in the cell cycle state (121). In proliferating cells, miRNAs downregulate translation and in cells in the arrested phase of the cell cycle miRNAs activate translation (121). Therefore, in ACC, where the proportion of cycling tumor cells has been found to be as low as 0.3% (122), miRNAs may in fact be responsible for increased KLK14 expression. We can then hypothesize

that KLK14 may inhibit its own transcription in a negative feedback loop as it becomes more abundant. Bayes et al. have shown that KLK6 activity is regulated by a negative feedback inhibition mechanism (62). However, this occurs post-translationally through autolytic cleavage (62). To our knowledge, a negative feedback loop that has control on kallikrein transcription has yet to be described, and further studies are needed to either confirm or dispute its existence.

The mRNA and protein concentrations of KLK1 and KLK11 in NSGTs have previously been studied (63, 123). However, their abundance in salivary gland tumors has not been confirmed until now. In our study, we found *KLK1* and *KLK11* to be downregulated in ACC compared to NSGT.

Few studies have detailed coordinated changes in *KLK1* and *KLK11* expression in either normal physiology or disease. Komatsu et al. demonstrated abundant co-expression of *KLK1* and *KLK11* compared to other members of the kallikrein family in normal human skin (124). In addition, increased expression of both KLK1 and KLK11 has been observed in high-grade renal cell carcinoma compared to low-grade renal cell carcinoma using immunohistochemistry (89, 125). Nonetheless, the parallel co-expression of *KLK1* and *KLK11* is an uncommon finding, and we are the first to document its occurrence in salivary gland tumorigenesis.

Studies looking only at the *KLK1* gene have shown that single nucleotide polymorphisms (SNPs) are associated with coronary artery stenosis (126), cerebral hemorrhage (127) and essential hypertension (128). Kontos et al. have suggested that SNPs of kallikrein genes possess value as putative genomic biomarkers (50). Evidence supporting this statement stems from research studying the role of PSA in prostate

cancer. Morote et al. found that SNPs of *KLK2* helps predict biochemical recurrence after radical prostatectomy (129). The presence and/or significance of kallikrein SNPs in salivary gland tumors have not been studied to date. It is possible that they may prove to be useful biomarkers; therefore, this hypothesis should be studied further.

Accumulating evidence indicates that alterations in *KLK11* expression are seen in a number of malignant tumors. Increased *KLK11* mRNA levels have been observed in prostate (130) and ovarian cancer (131) while decreased mRNA levels have been reported in testicular (85) and laryngeal cancer (88). In most cases, the differential expression pattern of *KLK11* in cancers has been documented to occur in conjunction with other members of the kallikrein family. Planque et al. highlighted this observation in non-small cell lung carcinoma (132). They found that KLK11 and six other kallikreins, including KLK4, KLK8, KLK10, KLK12-14, are more accurate in diagnosing lung cancer when considered together, as a multiparametric panel, rather than individually (132).

The role of kallikreins as cancer biomarkers has been questioned due to their lack of sensitivity and specificity in many tumors (133). However, the combination of multiple members of the KLK family, with or without other candidate biomarkers, may improve their clinical utility (50, 132, 133). Following the work of Planque et al. in lung cancer (132), a later study looked at the usefulness of a multiparametric panel in ovarian cancer (134). In that study, it was found that a combined panel of *KLK6, KLK13,* and *CA125* is more sensitive in detecting early stage cancer than *CA125* alone (134).

Previously, Darling et al. have suggested that individual kallikreins cannot be considered as specific markers for salivary gland tumors (95, 97, 99). Furthermore, if

KLKs are to be useful as biomarkers in salivary gland tumors multiparametric panels will need to be identified (98). 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 moving forward. If the co-expression of these four *KLKs* proves to be a valid multiparametric panel, it would be interesting to also study the expression of *c-kit* to see if it improves the panel's sensitivity and/or specificity compared to the *KLKs* alone. One anticipated challenge in the development of a multiparametric panel for the diagnosis of ACC is that the difference in ΔCq values between ACC and NSGT is quite small for all four kallikreins. Additionally, the expression levels of the four *KLKs* in the FFPE samples may not truly reflect those in the original tissues, which have not been subjected to heat, chemical exposure, and long-term storgage. Therefore, *KLK* expression will also need to be measured in fresh frozen samples. This will be discussed in more detail below.

The use of FFPE tissues for RT-qPCR is a feasible technique, which allows for the quantification of gene expression and the detection of disease-specific biomarkers (135, 136). A number of studies have successfully confirmed that extracted gene transcripts can be processed in this manner (137-139). Therefore, given the large archival collections of FFPE tissue samples worldwide the gene expression of countless diseases can be easily studied retrospectively. However, some researchers have questioned the accuracy of the quantitative analysis due to concerns about RNA degradation within the embedded specimens over time and the ill effects of delayed formalin fixation on RNA due to its short half-life (135, 140). Godfrey et al. previously

showed that only 3% of RNA from fixed samples is available for reverse transcription, which was reflected in higher C_T values by ~5 cycles in fixed compared to fresh tissues (140). For this reason, the expression of target genes must be normalized to a housekeeping gene if their levels are to be compared between specimens (135, 136). This process is thought to eliminate the effects of differences in RNA degradation between samples, but it does assume that the RNA of target and housekeeping genes degrade at the same rate within a particular sample (136).

This study is the first to quantify kallikrein expression in ACC and NSGT from FFPE extracts. As previously discussed, 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 methodology accounts for the difference in RNA degradation between specimens, it does overlook the fact that the measured RNA levels may not reflect the actual expression levels in the tissues before fixation (140). Therefore, the development of a future multiparametric panel for ACC should ideally use fresh tissues to maximize its accuracy.

Kallikreins have been identified in numerous biological fluids (63). However, KLK1, KLK8, KLK11, and KLK14 have only been observed together in saliva and cervicovaginal fluid (63). In this study, saliva from patients with ACC and NSGT was not available. In the future, it would be interesting to look at the expression levels of *KLK1*, *KLK8*, *KLK11*, and *KLK14* in both saliva and tissue specimens in patients with ACC. Planque et al. previously found the concentration of KLK11 to be upregulated in the serum of patients with non-small cell lung carcinoma and downregulated at the mRNA level in the cancerous lung tissue (132). They speculated that KLK11 was being

leaked into circulation due to the destruction of lung tissue and angiogenesis (132). Therefore, it is possible that the concentrations of KLKs may be increased in the saliva of patients with ACC and can be used for cancer detection. This could reduce the need for biopsies and help overcome some of the shortfalls of FNA in the diagnosis salivary gland tumors.

A limitation of this study is that all of the NSGT samples were taken from minor salivary glands. In contrast, some of the ACC samples were from major salivary glands. It is possible that this may have impacted the observed differences, or lack thereof, in the expression levels of each *KLK* between the two tissue types. Evidence supporting this speculation comes from previous work in our laboratory that showed higher expression of KLK6 in normal major glands compared to normal minor glands (95). Therefore, moving forward attempts should be made to compare *KLK* expression levels between minor and major salivary glands for both ACC and NSGT to determine any effects it may have on the results of this study.

Lastly, future studies need to include tissue samples with inflammation present within the glandular tissue. Although we excluded these samples in this study to help limit the effects of inflammation on kallikrein expression, this may not truly reflect salivary gland tumors in clinical practice. The impact this may have on the pattern of *KLK* expression in ACC remains unknown, however, one can postulate that inflammation will confound *KLK* levels in much the same way inflammation impacts PSA levels in prostate tissue.

Chapter 6

6   Conclusion

In this study, we looked at the gene expression of kallikreins in ACC and NSGT. 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 NSGT. 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 decreased 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 of ACC.

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Curriculum Vitae

Name: Zachary Ryan Kerr

Post-secondary St. Francis Xavier University
Education and Antigonish, Nova Scotia, Canada
Degrees: 2002-2005, BSc

        Dalhousie University
        Halifax, Nova Scotia, Canada
        2006-2010, DDS

        The University of Western Ontario
        London, Ontario, Canada
        2010-2011, General Practice Dental Residency

        The University of Western Ontario
        London, Ontario, Canada
        2012-2016, MD

        The University of Western Ontario
        London, Ontario, Canada
        2011-2017, Oral and Maxillofacial Surgery Residency

Honours and Undergraduate Student Research Award (USRA)
Awards   University of New Brunswick, 2003

        Undergraduate Student Research Award (USRA)
        St. Francis Xavier University, 2004

        Undergraduate Research Award (URA)
        St. Francis Xavier University, 2005

        The Leonard Goldfarb Price
        Dalhousie Dentistry, 2007

        Atlantic Society of Periodontology
        Dalhousie Dentistry, 2008

        Dr. David Manual Memorial Prize
        Dalhousie Dentistry, 2008

        Charles Bell Memorial Prize
        Dalhousie Dentistry, 2008

Dr. Sydney D. Campbell Bursary
Dalhousie Dentistry, 2009

Gaum-Brayton Endodontics Scholarship
Dalhousie Dentistry, 2009

Nova Scotia Dental Association Prize
Dalhousie Dentistry, 2009

Dr. Terry Ingham Memorial Scholarship
Dalhousie Dentistry, 2009

The American Association of Endodontists Prize
Dalhousie Dentistry, 2010

Dr. Frank Woodbury Memorial Prize
Dalhousie Dentistry, 2010

Dr. Ronald E. Warren Award
Canadian Association of Oral and Maxillofacial Surgery, 2012

Outstanding Scientific Abstract, 3rd Place
American College of Oral and Maxillofacial Surgeons, 2015

Related Work Experience:

Research Student, Supervisor: Dr. Allan Adam
University of New Brunswick, 2003

Research Student, Supervisor: Dr. Truis Smith-Palmer
St. Francis Xavier University, 2003, 2004

Teaching Assistant
St. Francis Xavier University, 2004