Kallikrein-related peptidase 10 expression in salivary gland tissues and tumours

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ABSTRACT

Objectives: Kallikrein-related peptidase 10 (KLK10) has been implicated in the development of several types of cancer. The purpose of this study was to analyze the expression of KLK10 in 3 types of salivary gland tumour and normal salivary glands.

Materials and methods: A standard immunoperoxidase staining technique was used to assess the immunoexpression profile of KLK10 in normal salivary glands and 3 types of salivary gland tumour: pleomorphic adenoma, adenoid cystic carcinoma and mucoepidermoid carcinoma.

Results: Pleomorphic adenomas showed significantly lower KLK10 levels than control tissues. Neither of the malignant tumours (adenoid cystic carcinoma and mucoepidermoid carcinoma) showed a significant alteration in the immunoreactive scores of KLK10 in comparison with the normal salivary gland tissues. KLK10 immunoreactive scores were comparable in adenoid cystic carcinoma and mucoepidermoid carcinoma. Pleomorphic adenoma had significantly lower levels of KLK10 than mucoepidermoid carcinoma.

Conclusions: The finding of lower KLK10 levels in pleomorphic adenoma suggests aberrant expression in a tumour that develops primarily from myoepithelial cells. A kallikrein cascade may play a role in the development and/or outcome of some salivary gland tumours.

Key words: Immunoexpression profile, Kallikrein-related peptidases, Salivary gland tumours

Received: May 17, 2012; Accepted: November 22, 2012

INTRODUCTION

Kallikrein-related peptidases (KLKs) are a family of 15 highly conserved trypsin- or chymotrypsin-like serine proteases encoded by genes KLK1-15 on chromosome 13 of the human genome (1). Multiple KLK genes are co-expressed in many different types of human tissue and are frequently co-ordinately deregulated in disease states, indicating a regulatory role in those tissues (2-6). KLKs are activated in complex proteolytic cascades that regulate both normal and pathological processes, such as semen liquefaction, skin desquamation, innate immunity and degradation and remodelling of extracellular matrix (ECM) (1). KLK3, also known as prostate specific antigen (PSA), has received the most attention as a valuable tumour marker for the screening, diagnosis, staging and monitoring of prostate cancer, and currently has the greatest impact on clinical practice (2-6). In addition, many other KLKs are aberrantly expressed in several different types of cancer and are useful biomarkers for cancer diagnosis, prognosis and monitoring (1, 4).

Experimental evidence indicates that KLKs may influence cancer-cell growth, angiogenesis, invasion and metastasis by proteolytic processing of growth factor-binding proteins, activation of growth factors and other proteases, release of angiogenic or anti-angiogenic factors, and degradation of ECM components (2-4, 6). However, whether KLKs exert cancer-promoting or cancer-inhibiting activities might depend on the tissue type and the tumour microenvironment (4). Since most evidence available has
been based on in vitro studies, more clinical studies are needed to examine the roles of KLKs in cancer biology (2-4, 6, 7).

KLK10, among other KLKs, was reported to be strongly expressed in the islets of Langerhans in the pancreas and may regulate the pro-hormone activation of insulin, glucagon, somatostatin and pancreatic polypeptide (2-4, 6-11). KLK10 has been shown to be upregulated in colon cancer cells (12), and is involved in testicular, breast and colorectal cancer (13-15). It has also been shown, along with other kallikreins, to be regulated by vitamin D3 in normal keratinocytes, which implicated it with KLK6 in skin carcinogenesis (16). In addition, KLK10 and KLK6 co-expression have been found to be indicators of poor prognosis in pancreatic ductal adenocarcinoma (17).

Salivary gland (SG) tumours are relatively uncommon, comprising less than 0.5% of all malignancies and 2-5% of all head and neck malignancies worldwide (18-20), but the impact of SG cancer is possibly misrepresented by the relatively low incidence rates. We have reported the expression of several KLKs in normal SGs and SG tumours (21-25), which suggests a potential for the utility of KLKs as biomarkers in SG neoplasms.

Because of the association of KLK10 with colon, pancreatic and skin cancer, this study aimed to determine whether KLK10 is expressed in SG tissues and SG tumours, and whether it was differently expressed in different types of SG malignancy.

MATeRIALS AND METHODS

Study design

Consecutively obtained SG tumours and normal tissues were utilised for this retrospective study, using specimens collected over a period of 7 years. For clinical correlation, age, gender, clinical stage, tumour grade and type of gland (major or minor) were recorded. Based on the Armed Forces Institute of Pathology (AFIP) and World Health Organization (WHO) guidelines, the cribriform and tubular patterns of adenoid cystic carcinoma (ACC) were classified as low-grade tumours whereas tumours showing a solid pattern were classified as high grade (26, 27). Patients with disease of all 4 clinical stages (I-IV) and all 3 tumour grades (high and low for ACCs; high, intermediate and low for mucoepidermoid carcinomas [MECs]) were represented in this study. Due to the small number of high-grade tumours, the intermediate- and high-grade tumours were combined into one category, called “intermediate/high” grade. Similarly, stages I and II were combined into the “low-stage” category, and stages III and IV were combined into the “high-stage” category and this modified stage was used in the clinical correlation tests with KLK levels.

Specimen characteristics

Archival formalin-fixed, paraffin-embedded tumour tissues consecutively obtained over a period of 7 years were retrieved from the Division of Oral Pathology, Department of Pathology, University of Western Ontario. A total of 39 SG tumours (13 cases of ACC, 9 cases of MEC and 17 cases of pleomorphic adenoma [PA]) and 10 normal SG controls (3 parotid, 3 submandibular and 4 minor glands) were used in the study. Each tumour specimen also contained associated normal gland tissue. Six MECs, 4 ACCs and 4 PAs arose within the parotid gland – all the rest arose within minor glands. The tumours were diagnosed according to criteria published in the Atlas of Tumour Pathology of the AFIP and the WHO (26, 27). Appropriate matching negative controls (primary antibody omitted) and positive controls (skin) were used. Ethical approval was obtained from the Office of Research Ethics, University of Western Ontario. This study was done in accord with the Helsinki Declaration (2002).

Assay method

A KLK10-specific rabbit polyclonal antibody raised against full-length recombinant KLK10 protein produced in yeast and tested for specificity and sensitivity, as previously described (28, 29), was used. The staining procedures followed, as formerly reported (18), included deparaffinization in xylene at room temperature (RT) followed by rehydration by transfer through graded alcohols. Endogenous peroxidase activity was blocked with fresh 3% H2O2 in methanol. Antigen retrieval was achieved by immersing the slides in boiling citrate buffer (pH 6.0) at 125°C under pressure in a decloaking chamber. Slides were blocked in 10% horse serum (in phosphate-buffered saline [PBS]) for 30 minutes at RT in a humidified chamber, then incubated with the KLK10 primary rabbit polyclonal antibody in horse serum (1:1600) overnight at 4°C. Anti-rabbit immunoglobulin conjugated with hors eradish peroxidase micro-polymer (ImmunPRESS Reagent Kit; Vector laboratories, Burlingame, CA, USA) was applied for 30 minutes at RT. After rinsing with PBS, the enzymatic reaction was developed in a freshly prepared solution of 3,3’-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, Oakville, Ontario, Canada) for 5 minutes. The sections were then counterstained with hematoxylin, dehydrated in graded alcohol solutions, cleared with xylene and mounted in Cytoseal mounting medium (Richard-Allan Scientific, Kalamazoo, MI, USA).

A proportion score and intensity score using a previously documented system was utilized to assess KLK10 immunostaining (21, 30). The proportion score represents the estimated fraction of positively staining tumour cells (where 0 = none; 1 = 1/100; 2 = 1/100 – 1/10; 3 = 1/10 – 1/3; 4 = 1/3 – 2/3; 5 > 2/3). For staining intensity, the score...
Statistical analysis

The non-parametric Wilcoxon signed-rank test and the Spearman rank correlation tests were used, where appropriate, for the statistical analyses. Statistical significance was set at $p \leq 0.05$.

RESULTS

Cellular localization of KLK10 in SG tissues

In this study, all SG tumours and normal SG tissues demonstrated immunoreactivity to KLK10, which was confined to the cellular cytoplasm of both ductal and non-ductal cells (Fig. 1). For the purpose of this study, the cytoplasm referred to the cytosolic component of the cell, including the cytoplasmic organelles and the mucous globules (in mucous cells) within the plasma membrane, exclusive of the nucleus. No KLK10 staining was observed in the connective tissue stroma of the SGs and the SG tumours – this was not surprising as KLKs are produced by glandular cells, and are not necessarily secreted into the stroma of glands or tumours. Ductal structures of the normal SGs and all 3 types of tumour showed strong positivity in the inner (luminal) layer of the ductal wall.

is represented by the estimated average staining intensity of positively staining tumour cells (where 0 = none; 1 = weak; 2 = intermediate; 3 = strong). The overall amount of positive staining, called the immunoreactive score (IRS), was then expressed as the sum of the proportion and intensity scores (ranges = 0 for negative staining and 2-8 for positive staining).

Ductal and acinar cells of normal SG tissues as well as the duct-like structures and non-ductal structures of SG tumours were scored separately. In tumour tissue, cells lining duct-like structures and non-ductal cells were scored for extent of positive and intensity of staining. In this study, duct-like cells are defined as those cells that line the lumens of duct-like structures within tumour tissue, while non-ductal cells are any cells in the tumour tissue that are not obviously lining ducts. Squamous, mucous, and intermediate cells of the MEC were scored separately. These cells were identified by 2 experienced histopathologists, based on morphological descriptions in the Atlas of Tumour Pathology of the AFIP (27). The staining was assessed by 3 trained examiners to achieve consistency by comparison of assessments, interpreting the positive control as a reference intensity score of 3.
KLK10 in salivary gland tumours

In normal SGs, both mucous and serous secretory acinar cells showed consistent positive cytoplasmic staining for KLK10, although the intensity of staining was generally higher in serous than in mucous cells. Consistent with previous findings (31, 32), staining of myoepithelial cells in normal SG tissues was not detected. Likewise, the duct epithelium of the major and minor SGs was also consistently positive for all of the KLKs. Unlike all previously studied KLKs (KLK6, KLK7, KLK8, KLK13 and KLK14), all of which showed higher staining intensity in ductal than acinar cells, KLK10 was an exception and there was no obvious difference in the staining intensity between ductal and acinar cells (Fig. 1A).

Expression of kallikreins in SG tumours

In PA, immunoreactivity to KLK10 was observed in both the myoepithelial and ductal cells, where ductal cells scored higher than non-ductal cells (Fig. 1B). However, it was occasionally noted that the myoepithelial cells in myxoid areas of the PA showed remarkably stronger positivity than ductal cells. The tubular, cribriform and solid types of ACC stained positively for KLK10. Both the non-ductal and ductal cells of the ACC tissues showed staining for KLK10 (Fig. 1C), although generally stronger staining was seen in the ductal cells. It is worth noting that the scarcity of cytoplasm in the cells comprising the solid form of ACC, however, made it difficult to score the staining intensity and extent. In MEC, immunoreactivity to KLK10 was also observed in squamous (IRS=6.6), intermediate (IRS=6.4) and mucous cells (IRS=6.0). The staining of mucous cells, which appeared as a faint, homogenous tint of brown throughout the mucous globules, was generally less intense than the staining of squamous and intermediate cells (Fig. 1D).

The KLK10 levels in PA, ACC and MEC were compared with the levels in normal tissue. Neither of the malignant tumours (ACC and MEC) showed a significant alteration in the IRS levels of KLK10 in comparison with its levels in the normal SG tissues, although ACC showed slightly less KLK10. PA tissues, however, showed significantly lower KLK10 levels than control tissues (IRS 5.00 vs 6.00, p=0.009).

KLK10 IRS levels were comparable in ACC and MEC. Although KLK10 IRS levels were lower in PA than ACC, the difference in expression between the 2 tumours only approached significance. PA and MEC showed significantly different IRS levels of KLK10, which were lower in PA than in MEC (IRS 5.00 vs 6.30, p=0.007).

No statistical association was found between KLK10 expression and tumour grade or stage.

Kallikrein expression profile

The results showed no correlation between the KLKs in normal SGs and the SG tumours, other than in ACC (Fig. 2). Statistical analysis showed a significantly
strong association between the IRS levels of KLK7, KLK10 and KLK14 in ACC. Although the IRS of KLK7 in ACC tissues was moderately associated with the IRS of KLK10 \( (r=0.616, p=0.025) \), there was a stronger association between the IRS of KLK7 and KLK14 in the same tumour (Pearson’s \( r=0.802, p=0.001 \)). Similarly, the association between KLK7 and KLK14 was stronger than that of KLK10 and KLK14 \( (r=0.785, p=0.003) \) (Tab. I).

**TABLE I - ASSOCIATION AMONG IRS LEVELS OF DIFFERENT KLKs IN ACCs AS DETERMINED BY PEARSON’S CORRELATION COEFFICIENT (R VALUE) AND P VALUES (IN BRACKETS)**

<table>
<thead>
<tr>
<th>KLK</th>
<th>KLK7</th>
<th>KLK8</th>
<th>KLK10</th>
<th>KLK13</th>
<th>KLK14</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLK6</td>
<td>0.494(0.073)</td>
<td>0.144 (0.622)</td>
<td>0.310 (0.303)</td>
<td>0.272 (0.347)</td>
<td>0.211 (0.489)</td>
</tr>
<tr>
<td>KLK7</td>
<td></td>
<td>0.441 (0.115)</td>
<td>0.616 (0.025)*</td>
<td>0.405 (0.151)</td>
<td>0.802 (0.001)*</td>
</tr>
<tr>
<td>KLK8</td>
<td></td>
<td></td>
<td>0.205 (0.501)</td>
<td>0.423 (0.132)</td>
<td>0.239 (0.432)</td>
</tr>
<tr>
<td>KLK10</td>
<td></td>
<td></td>
<td></td>
<td>0.131 (0.670)</td>
<td>0.785 (0.003)*</td>
</tr>
<tr>
<td>KLK13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.208</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.496)</td>
</tr>
</tbody>
</table>

* Significant at \( p=0.05 \)

IRS, immunoreactive score; KLK, kallikrein-related peptidase; ACC, adenoid cystic carcinoma

**DISCUSSION**

*Kallikreins in SGs*

Several studies have revealed the presence of most KLKs, including KLK10, in SGs at the protein level, by immunohistochemistry and ELISA, and at the nucleic acid level by PCR (29, 31, 32). The relative expression levels of KLK10 in the normal SGs observed in our study were in general agreement with the mRNA findings in the study by Shaw and Diamandis in 2007 (32). The expression of the KLK family in the normal SG tissues may mean that they function in the SGs in a novel proteolytic cascade. Interestingly, KLK10 showed equal immunoeexpression in mucous acinar cells and ductal cells but more intense positivity in serous acinar cells in normal SGs. This may be due to production of more mucoproteins in mucous and ductal cells than in serous cells.

*Kallikreins in SG tumours*

We have previously reported the IRS of KLK6, KLK8, KLK13 and KLK14, and the absence of KLK3 (PSA) and KLK5, in several benign and malignant SG tumours (21-25, 33). It is possible that the expression of some KLKs may be dysregulated in benign and malignant SG tumours and may consequently represent clinical tumour biomarkers.

Strong positivity of KLK10 was observed in the normal SGs, confirming the results of previous studies. As the levels of KLK10 in MEC were comparable to normal, this may suggest the absence of a role for KLK10 in the carcinogenesis of MEC. However, the finding of lower KLK10 levels in PA and ACC points to a possible role for KLK10 in inhibiting the

**Relationship between kallikreins and clinical parameters**

The relationship between the IRS of KLK10 and several clinical parameters pertaining to the malignant tumours was examined. Patient age at diagnosis ranged from 39 to 85 years for ACCs and from 30 to 84 years for MECs, with a mean age at presentation of 61.7 and 56.4 years, respectively. Due to the small sample size of the individual ACC and MEC cases, the cases were grouped together and the association tests carried out on the combined malignant tumour group. In these correlation tests, statistical significance was assessed at the \( p=0.05 \) level. Thus, the modified histological grade consisted of 2 groups: the “intermediate/high-grade” group and the “low-grade” group (as stated in Methods). No significant difference was found in KLK10 IRS levels between these 2 groups.

The tumours were classified into stages I-IV; however, due to the small sample size, tumours classified as stages I and II were combined into the “low-stage” group whereas those classified as stages III and IV were merged into the “high-stage” group (as stated in Methods). As previously described for histological grade, the levels of each of the KLKs in low-stage and high-stage tumours were compared. There was no statistical association between the IRS levels of KLK10 and modified tumour stage.

KLK10 showed significantly higher IRS levels in the SG tumours arising in males than those arising in females (IRS 6.67 vs 6.00, \( p=0.041 \)). Patient age showed a weak negative association with KLK10 \( (r=-0.106, p=0.639) \), which was not statistically significant. KLK10 showed comparable IRS levels in tumours arising from both major and minor SGs.
The expression patterns of KLK7 and KLK14 in the skin desquamation cascade through the processing of DSG1 (4). KLK7 and KLK10 are both reportedly co-expressed and upregulated in Alzheimer's disease, where they also showed a strong positive correlation (43). Apart from their co-expression in normal human tissues, there is no other report of KLK7 and KLK10 co-expression with pathological relevance in the current literature. Several studies have shown the co-expression of KLK10 and KLK14 in several normal human tissues, including the SGs, skin and lungs (32). Nonetheless, there has been no definitive implication of both KLK10 and KLK14 in a proteolytic cascade operating in a pathological condition.

The expression patterns of KLK7 and KLK14 suggest they may have a role in promoting the growth of myoepithelial cell tumours, such as ACC. We have also considered that KLK10 may confer a protective phenotype on normal SG tissues, as evidence from its lower expression in the tumours. Based on our findings, it appears that the regulation of KLK7, KLK10 and KLK14 in PA and ACC, both composed largely of myoepithelial cells, are linked. Hence, it is likely that the tumourigenesis of myoepithelial cell neoplasms is associated with one or more as yet unidentified proteolytic pathways involving KLK7, KLK10 and KLK14 operating in these tumours. As this is the first report of such a cascade, it would be interesting to examine other SG tumours containing myoepithelial cells, such as myoepitheliomas, for the expression of these 3 KLKs and the presence or absence of a similar correlation.

Ruckert et al (17) reported that co-expression and upregulation of KLK6 and KLK10 may be indicators of poor prognosis in pancreatic ductal adenocarcinoma. We did not find such a correlation in the current study, but the co-expression of KLK6 and KLK10 in mucoepidermoid carcinomas may be worth further investigation.

Our previous studies indicate that KLK6, KLK7, KLK8, KLK13 and KLK14 do not show a gender predilection in malignant SG tumours. In contrast, the current study revealed significantly higher KLK10 immunoeexpression in the SG malignancies of male subjects than in those of female subjects. Although the biological significance of this finding is unknown, this differential expression of KLK10 may imply a gender-specific role for this KLK and may suggest a potential role for KLK10 as a cancer biomarker in male patients suffering from SG cancer. However, further studies are needed to reproduce this finding, which may be due to stochastic effects.

To conclude, it appears that panels of selected KLKs may be promising new biomarkers in SG tumours; however, further studies are needed to quantitatively measure their expression and to correlate with prospective clinical data, for individual tumours. Some kallikreins are relatively highly expressed in SG tissues. This may mean that within SGs, a kallikrein cascade is functional but the nature of this function is yet to be clarified. In addition, a similar profile is noted within SG tumours but for these, too, a functional role in tumour growth and progression, as well as prognosis, has yet to be identified.

ACKNOWLEDGMENTS

The authors would like to thank Mrs Linda Jackson-Boeters for her technical support and advice.

Financial support: This study was funded by the Schulich School of Medicine and Dentistry Internal Grant. Dr Eleftherios P. Diamandis donated the primary antibodies.

Conflict of interest statement: The authors declare no conflict of interest

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