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Human kallikrein 13 expression in salivary gland tumors

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ABSTRACT: The human kallikrein 13 protein (hK13) is expressed in many normal tissues. Petraki et al have previously described presence of hK13 in salivary gland tissue, localized to duct epithelia and some acinar cells. The aim of this study was to determine whether hK13 is expressed in salivary gland tissues and salivary gland tumors (both benign and malignant), in order to compare normal with tumor tissues. Pleomorphic adenomas (PA), adenoid cystic carcinomas (ACC), polymorphous low grade adenocarcinomas (PLGA), acinic cell carcinomas (ACI), mucoepidermoid carcinomas (MEC) and adenocarcinomas not otherwise specified (ANOS) of both minor and major salivary glands were examined. The results of this study indicate that most salivary gland tumors show high levels of expression of hK13. Overall, staining in PA was significantly less than that seen in normal salivary gland tissue. PLGA, ACC and ANOS each stained significantly more than normal salivary gland tissue while MEC and ACI did not. Ductal cells and cells lining duct-like structures showed a higher intensity of staining than non-ductal cells in most tumors. Tumors which exhibited only non-ductal cells also exhibited cytoplasmic staining. In conclusion, we demonstrate the high expression of hK13 in several common salivary gland tumors. (Int J Biol Markers 2006; 21: 106-10)

Key words: Kallikreins, Human kallikrein 13, Salivary gland tumors, Prognostic markers, Immunohistochemistry

INTRODUCTION

Human tissue kallikreins (hKs) are a subfamily of serine proteases encoded by 15 genes, localized in tandem on human chromosome 19q13.4 (1-4). Recently, many novel kallikrein genes have been characterized and a detailed map of the human kallikrein gene locus has been constructed (4). Kallikreins were originally described as enzymes that cleave vasoactive peptides (kinins) from kininogen but only a few of the family meet this criterion (5). The members of the kallikrein family are now implicated in a wide range of normal and pathological processes, either independently or as part of a proteolytic cascade (4, 6).

Numerous studies have shown that kallikreins are overexpressed in ovarian, breast and prostatic carcinomas and that some may be important new biomarkers for diagnosis and monitoring of many cancer types (7, 8). The overexpression of kallikreins in malignant tumors has been linked with both favorable and poor patient prognosis (4).

Several studies have shown that most human kallikreins are expressed in the salivary glands (1, 2, 9, 10). It is thus possible that some members of this family may be valuable markers for differential diagnosis, subtyping and monitoring of patients with salivary gland carcinomas. The hK13 protein (hK13), encoded by the kallikrein 13 gene (*KLK13*), is expressed in many normal tissues. Petraki et al have described the presence of hK13 in salivary gland tissue, localized to duct epithelia and some acinar cells (10). To date, there are a few reports linking hK13 to cancer (11, 12). Kapadia et al showed that hK13 was able to cleave the major components of the extracellular matrix and may play a role in tissue remodeling and/or tumor invasion and metastasis (12). Our own studies have shown that hK6 is expressed in salivary gland tumors, although apparently down-regulated when compared to normal salivary gland tissues (13).

The aim of this study was to determine whether hK13 is expressed in salivary gland tissues and salivary gland tumors (both benign and malignant), in order to compare normal with tumor tissues. This is the first report on hK13 expression in salivary gland tumors.

METHODS

Archival formalin-fixed, paraffin-embedded tumor tissues from the Division of Oral Pathology, Department of Pathology, University of Western Ontario were cut in 5-micron sections and stained using a standard immunoperoxidase technique. Twenty-six pleomorphic

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adenomas (PA), 23 adenoid cystic carcinomas (ACC), 13 polymorphous low grade adenocarcinomas (PLGA), 7 acinic cell carcinomas (ACI), 24 mucoepidermoid carcinomas (MEC), 8 adenocarcinomas not otherwise specified (ANOS) of salivary gland origin and 57 normal salivary gland controls were used in the study. The tumors were diagnosed according to criteria published in the *Atlas of Tumor Pathology: Tumors of the Salivary Glands* of the Armed Forces Institute of Pathology (14). Appropriate matching negative controls (primary antibody omitted from tissue slide), one for each experimental tumor section, were used.

An hK13-specific rabbit polyclonal antibody raised against full-length recombinant hK13 protein produced in yeast was used at a dilution of 1:4000. The recombinant hK13 protein was produced and purified by HPLC as described previously (15).

Staining procedures included deparaffinization in xylene for 13 minutes with two changes of xylene at room temperature (RT) followed by transfer through graded alcohols and rehydration. Endogenous peroxidase activity was blocked with fresh 3% H_2O_2 in methanol for 5 minutes. The sections were rinsed in PBS for 10 minutes on a shaker. Antigen retrieval was achieved by immersing the slides in boiling citrate buffer (pH 6.0) for 10 minutes at high power, and 10 minutes 50% power in a microwave oven. They were then rinsed in water and PBS for 5 minutes, blocked in 10% horse serum for 30 minutes at RT in a humidified chamber, and incubated with the hK13 primary rabbit polyclonal antibody for one hour at RT. After two washes in PBS, the biotinylated goat anti-rabbit secondary antibody (1:200 dilution, prepared in 10% horse serum, Vector Elite Kit, Vector Laboratories, Burlington, Ontario) was applied for 30 minutes at RT. After two rinses with PBS, the freshly prepared ABC reagent was applied for 30 minutes at RT. The enzymatic reaction was developed in a freshly prepared solution of 3, 3′-diaminobenzidine tetrahydrochloride (Sigma Aldrich, Oakville, Ontario) for 5 minutes. The sections were then rinsed with water, counterstained with hematoxylin for 3 minutes, dehydrated, cleared with xylene and mounted.

A proportion score and intensity score using a well-documented system were used to assess hK13 immunostaining (16, 17). The proportion score represents the estimated fraction of positively staining tumor 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 is represented by the estimated average staining intensity of positively staining tumor cells (where 0 = none; 1 = weak; 2 = intermediate; 3 = strong). The overall amount of positive staining was then expressed as the sum of the proportion and intensity scores (ranges = 0 for negative staining and 2-8 for positive staining). In normal salivary gland tissue, ductal and acinar cells were scored separately. In tumor tissue, cells lining duct-like structures and non-ductal cells were scored for extent of positivity and intensity of staining. For the purposes of this study, ductal cells are regarded as those cells that line the lumens of duct-like structures within tumor tissue, while non-ductal cells are any cells in the tumor tissue that are not obviously lining ducts. Cells lining pseudocystic spaces, such as those in ACC, were scored as non-ductal cells. For MEC, squamous cells, mucous cells and intermediate cells were scored separately. Non-epithelial cells were not scored. The staining was assessed by separate examiners to achieve consistency by comparison and correlation of assessments in order to reduce interexaminer variability.

Wilcoxon and Dunn's multiple comparisons tests were used, where appropriate, for the statistical analyses.

RESULTS

The hK13 immunoreactivity was assessed in the cytoplasm of cells that stained positively. In general, the normal mucous (Fig. 1A) and serous glands (Fig. 1B) and all the tumors showed a relatively high overall staining for both ductal and non-ductal cells, the latter being an exception in pleomorphic adenoma (Fig. 1C). These data are summarized in Table I.

Overall, staining in PA was significantly less than that seen in normal salivary gland tissue (5.79 vs 6.77; p=0.0019). The malignant tumors as a group stained significantly more than normal salivary gland tissue (7.18 vs 6.77; p=0.0026), and significantly more than PA (7.18 vs 5.79; p<0.0001) (Tab. II). PLGA (7.42 vs 6.77) (Fig. 1D), ACC (7.17 vs 6.77) (Fig. 1E) and ANOS (7.78 vs 6.77) (Fig. 1F) individually all stained significantly more than normal salivary gland tissue (p=0.0097, 0.0154 and 0.0007, respectively). MEC (Fig. 1G) and ACI (Fig. 1H) did express more hK13 than normal salivary gland tissue, but scores were not statistically significant because of the low "n" value for these tumors.

Analysis of hK13 by the different cell types also yielded some significant differences. PA ductal and non-ductal cells stained significantly less than normal gland ducts and non-ductal cells (7.00 vs 7.44; p=0.041 and 4.77 vs 6.11; p=0.0039, respectively). ACC non-ductal cells stained significantly more than normal gland non-ductal cells (6.96 vs 6.11; p=0.0061). PLGA non-ductal cells also stained significantly more than normal salivary gland non-ductal cells (7.15 vs 6.11; p=0.0074).

PA ductal cells stained significantly more than PA non-ductal cells (7.00 vs 4.77; p<0.0001). PA non-ductal cells also stained significantly less than malignant non-ductal cells (4.77 vs 6.96; p=0.0002). PLGA ductal and non-ductal cells stained significantly more than those of PA (7.69 vs 7.00; p=0.0241 and 7.45 vs 4.77; p<0.0005, respectively). ACC non-ductal cells also stained significantly more than PA non-ductal cells (6.96 vs 4.77; p=0.0002). In MEC, there was a significant difference in

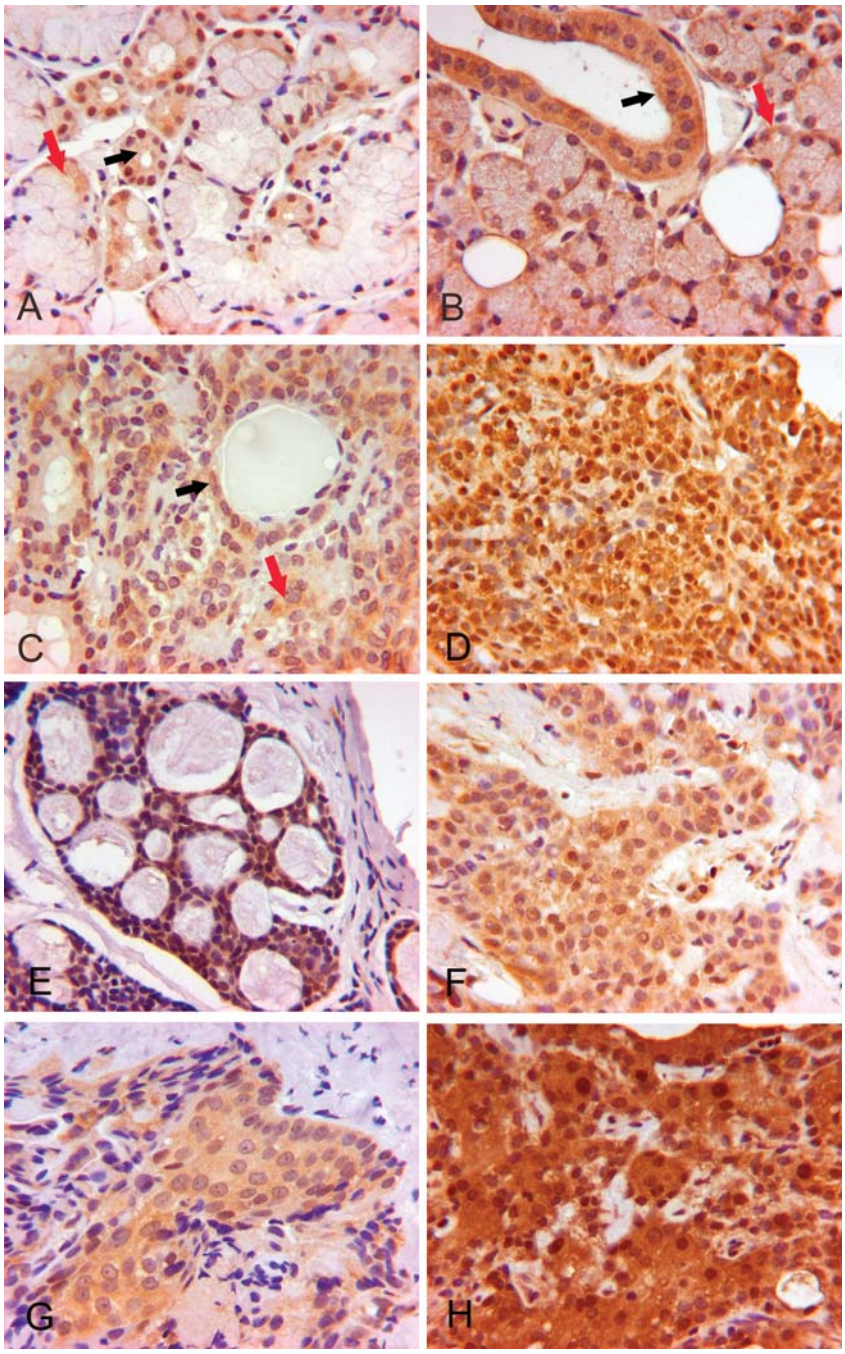

Fig. 1 - *hK13 staining (brown staining in cytoplasm of cells) is seen in:*
A. Normal mucous glands, showing intercalated duct (black arrow) and variable staining of mucous glands (red arrow) (original magnification x400).
B. Normal serous glands, showing excretory duct (black arrow) and staining of serous acini (red arrow) (original magnification x400).
C. Pleomorphic adenoma, showing ductal cells (black arrow) and cells of probable myoepithelial differentiation (red arrow) (original magnification x400).
D. Polymorphous low grade adenocarcinoma (original magnification x400).
E. Adenoid cystic carcinoma (original magnification x400).
F. Adenocarcinoma not otherwise specified (original magnification x400).
G. Mucoepidermoid carcinoma (original magnification x400).
H. Acinic cell carcinoma (original magnification x400).

hK13 expression in the different types of cells (p=0.004; see Tab. III); a multiple comparisons test showed that intermediate cells stained more than mucous cells (p<0.01) and more than squamous cells (p<0.05).

DISCUSSION

The kallikrein gene locus on chromosome 19q13.4 has been well characterized (1, 4). The family consists of 15 genes encoding for secreted serine proteases. Among the proteins encoded, prostate-specific antigen (PSA or hK3) has been shown to be a valuable marker for prostate cancer (18). A number of other kallikreins, such as hK5, hK6, hK7, hK8, hK10, hK11 and hK14, have also been associated with various forms of malignancy (1-4, 7). For example, the *KLK8* gene is up-regulated in ovarian cancer and its higher expression is associated with a favorable outcome (19, 20). hK13 itself has been associated with ovarian cancer (11). For these reasons, the possible role of kallikrein family members (hK13 in the current study) in cancer is worth investigating.

TABLE I - AVERAGE (SD[1]) STAINING IN DUCTAL CELLS AND NON-DUCTAL CELLS AND TOTAL SCORES FOR IMMUNOSTAINING OF SALIVARY TUMORS FOR HUMAN KALLIKREIN 13

	Ductal cells				Non-ductal cells				Paired average total scores
	n[3]	P[4]	I[5]	Total	n[3]	P	I	Total	
PA[2]	24	4.92 (0.28)	2.08 (0.83)	7.00 (0.98)	26	3.42 (1.50)	1.35 (0.75)	4.77 (2.14)	5.79
PLGA	13	4.85 (0.55)	2.85 (0.38)	7.69 (0.85)	13	4.62 (0.51)	2.54 (0.66)	7.15 (0.90)	7.42
ACC	23	4.78 (0.67)	2.61 (0.66)	7.39 (1.66)	23	4.52 (0.79)	2.43 (0.79)	6.96 (1.43)	7.17
ACI		Absent	Absent	Absent	7	5.00 (0.00)	2.29 (0.76)	7.29 (0.76)	7.28
ANOS		Absent	Absent	Absent	8	5.00 (0.00)	2.88 (0.35)	7.88 (0.35)	7.78
Normal gland	57	4.89 (0.49)	2.54 (0.63)	7.44 (0.93)	57	4.53 (0.98)	1.58 (0.82)	6.11 (1.42)	6.77

[1]For staining score definition, see text. SD = standard deviation
[2]Please see non-standard abbreviations
[3]Number of samples
[4]Proportion score
[5]Intensity score

TABLE II - AVERAGE[1] hK13 STAINING IN NORMAL GLANDS AND TUMORS

	Normal salivary gland (n = 57)	PA (n = 26)	All malignant tumors (n = 75)
Average (SD)	6.77 (0.89)	5.79 (1.44)	7.18 (0.95)

[1]Average represents the average of all the total scores, for both ductal and non-ductal cells

TABLE III - hK13 STAINING IN MUCOEPIDERMOID CARCINOMAS OF MAJOR AND MINOR GLANDS

n = 24	Squamous cells			Mucous cells			Intermediate cells		
	P	I	Total	P	I	Total	P	I	Total
Average	5.00	1.63	6.63	4.60	1.70	6.30	4.96	2.39	7.35
SD	0.00	0.81	0.81	0.94	0.80	1.34	0.21	0.66	0.78

Petraki et al have reported the expression of hK13 in normal salivary gland tissue, in both major (3 cases) and minor (3 cases) glands (10). Intense staining was seen in duct epithelia, with some positivity in acinar cells. This compares well with the current study, in which more intense staining was seen in duct epithelium than in non-ductal epithelium, although this study reports on a much larger number of salivary glands. The findings of this study are supportive of those of Petraki et al, who suggested that hK13 is a secreted protein (10).

The results of this study indicate that salivary gland tumors express high levels of hK13. In general, immunoexpression of hK13 was diffuse, but the intensity varied and differences were noted with individual types of cells. Ductal cells showed a higher intensity of staining than non-ductal cells in most tumors. In MEC, squamous and mucous cells showed a relatively low intensity of staining while intermediate cells showed a diffuse and higher intensity of staining, of which the meaning is not yet clear.

In this paper we report for the first time the high expression of hK13 in many salivary gland tumors. This is not unexpected, as hK13 is a secreted protein expressed in glandular tissues (10), but further studies are required to determine whether the *KLK13* gene is up-regulated in salivary gland tumors and to determine the correlation with clinical outcome. The measurement of serum levels of hK13 is required in order to assess whether it can be used as a serum marker to monitor salivary gland tumors. In this study serum from these patients was not available. Examination of other kallikreins in salivary gland tumors merits investigation, since almost all kallikreins are expressed in this tissue (1-4). hK13, together with the aforementioned kallikreins, may be part of an enzymatic cascade pathway which is operating in many tissues, including the salivary glands, skin, ovary, etc. (4, 6).

From these results it is apparent that hK13 cannot be considered a specific marker for any of the salivary gland tumors studied, but it has potential to be utilized with other markers. For most if not all cancers, panels of biomarkers are most useful for diagnosis, monitoring and prognosis. The pathobiological role of hK13 in salivary gland tissues also warrants further investigation.

In conclusion, we demonstrate the high expression of hK13 in several salivary gland tumors. This and other kallikreins have potential to be useful as biomarkers for salivary gland neoplasms.

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Abbreviations:
PA: pleomorphic adenoma
ACC: adenoid cystic carcinoma
PLGA: polymorphous low grade adenocarcinoma
ACI: acinic cell carcinoma
MEC: mucoepidermoid carcinoma
ANOS: adenocarcinoma not otherwise specified
hK13: human kallikrein 13
RT: room temperature
PBS: phosphate-buffered saline

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REFERENCES

1. Diamandis EP, Yousef GM, Luo LY, Magklara A, Obiezu CV. The human kallikrein gene family – implications in carcinogenesis. Trends Endocrinol Metab 2000; 11: 54-60.
2. Yousef GM, Diamandis EP. The new human tissue kallikrein gene family: Structure, function and association to disease. Endocr Rev 2001; 22: 184-204.
3. Borgono CA, Michael IP, Diamandis EP. Human tissue kallikreins: physiologic roles and applications in cancer. Mol Cancer Res 2004; 2: 257-80.
4. Borgono CA, Diamandis EP. The emerging roles of human tissue kallikreins in cancer. Nat Rev Cancer 2004; 4: 876-90.
5. Bhoola KD, Figueroa CD, Worthy K. Bioregulation of kinins: kallikreins, kininogens, and kininases. Pharmacol Rev 1992; 44: 1-80.
6. Yousef GM, Diamandis EP. Human tissue kallikreins: a new enzymatic cascade pathway? Biol Chem 2002; 383: 1045-57.
7. Diamandis EP, Yousef GM. Human tissue kallikreins: A family of new cancer biomarkers. Clin Chem 2002; 48: 1198-205.
8. Diamandis EP, Yousef GM. Human tissue kallikrein gene family: a rich source of novel disease biomarkers. Expert Rev Mol Diagn 2001; 1: 182-90.
9. James GK, Pudek M, Berean KW, Diamandis EP, Archibald BL. Salivary duct carcinoma secreting prostate-specific antigen. Am J Clin Pathol 1996; 106: 242-7.
10. Petraki CD, Karavana VN, Diamandis EP. Human kallikrein 13 expression in normal tissues: an immunohistochemical study. J Histochem Cytochem 2003; 51: 493-501.
11. Scorilas A, Borgono CA, Harbeck N, et al. Human kallikrein 13 protein in ovarian cancer cytosols: a new favourable prognostic marker. J Clin Oncol 2004; 22: 678-85.
12. Kapadia C, Ghosh MC, Grass L, Diamandis EP. Human kallikrein 13 involvement in extracellular matrix degradation. Biochem Biophys Res Commun 2004; 323: 1084-90.
13. Darling MR, Jackson-Boeters L, Daley TD, Diamandis EP. Human kallikrein 6 expression in salivary gland tumors. J Histochem Cytochem 2006; 54: 337-42. Epub 2005 Nov 14.
14. Ellis GL, Auclair PL. Atlas of Tumor Pathology: Tumors of the Salivary Glands. Washington, D.C.: Armed Forces Institute of Pathology, 1996; 39-56; 155-228.
15. Kapadia C, Chang A, Sotiropoulou G, et al. Human kallikrein 13: Production and purification of recombinant protein and monoclonal and polyclonal antibodies, and development of a sensitive and specific immunofluorometric assay. Clin Chem 2003; 49: 77-86.
16. Allred DC, Clark GM, Elledge R, et al. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. J Natl Cancer Inst 1993; 85: 200-6.
17. Tuck AB, O'Malley FP, Singhal H, et al. Osteopontin expression in a group of lymph node negative breast cancer patients. Int J Cancer 1998; 79: 502-8.
18. Stamey TA, Yang N, Hay AR, McNeal JE, Freiha FS, Redwine E. Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. N Engl J Med 1987; 317: 909-16.
19. Magklara A, Scorilas A, Katsaros D, et al. The human KLK8 (neuropsin/ovasin) gene: identification of two novel splice variants and its prognostic value in ovarian cancer. Clin Cancer Res 2001; 7: 806-11.
20. Kishi T, Grass L, Soosaipillai A, et al. Human kallikrein 8, a novel biomarker for ovarian carcinoma. Cancer Res 2003; 63: 2771-4.

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