

Human kallikrein 3 (prostate-specific antigen) and human kallikrein 5 expression in salivary gland tumors

M.R. Darling¹, S. Tsai², L. Jackson-Boeters¹, T.D. Daley¹, E.P. Diamandis³

¹Division of Oral Pathology, Department of Pathology, University of Western Ontario

²Faculty of Medicine & Dentistry, University of Alberta, Edmonton

³Department of Pathology and Laboratory Medicine, Mount Sinai Hospital and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto - Canada

ABSTRACT: The human kallikrein 5 protein (hK5) is expressed in many normal tissues, most notably in skin, breast, salivary gland and esophagus. It has also been shown to be a potential biomarker for breast, ovarian and testicular cancer. Human kallikrein 3 (hK3; prostate-specific antigen) is the most useful marker for adenocarcinoma of the prostate gland. The aim of this study was to determine whether hK3 and hK5 are expressed in salivary gland tissues and salivary gland tumors (both benign and malignant), in order to compare normal with tumor tissues. Pleomorphic adenomas, adenoid cystic carcinomas, polymorphous low-grade adenocarcinomas, acinic cell carcinomas, mucoepidermoid carcinomas and adenocarcinomas not otherwise specified of both minor and major salivary glands were examined. The results of this study indicate that most salivary gland tumors do not show high levels of expression of hK5. Staining was most prominent in keratinizing epithelia in pleomorphic adenomas. hK3 is not expressed in salivary gland tumors. (Int J Biol Markers 2006; 21: 201-5)

Key words: Kallikreins, Human kallikrein 3, Human kallikrein 5, 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). Many members of the kallikrein family are implicated in a wide range of normal and pathological processes, either independently or as part of a proteolytic cascade (4, 5).

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 (6-8). 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). 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.

hK3 (prostate-specific antigen, PSA) is the best known cancer biomarker and has been widely used in screening for early detection of prostate cancer in the last decade (10). PSA is known to be a kallikrein (11) and has been demonstrated in salivary gland tissues by means of in situ hybridization (12). James et al reported expression of hK3 in a salivary duct carcinoma (9).

The kallikrein 5 gene (*KLK5*) was initially cloned by Yousef and Diamandis as the kallikrein-like gene, *KLK-L2* (13). The hK5 protein is expressed in many normal tissues, most notably in skin, breast, salivary gland and esophagus, and to a lesser extent in numerous other tissues. To date, there are a few reports linking hK5 to cancer. It may be a biomarker and prognostic indicator for breast, ovarian and testicular tumors (14-17).

The aim of this study was to determine whether hK3 and hK5 are 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 of which we are aware on hK3 and hK5 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 into 5-micron sections and stained using a standard immunoperoxidase technique. Twenty-six pleomorphic 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 (18). Appropriate matching negative controls (primary antibody omitted from tissue slide), one for each experimental tumor section, and positive controls (prostate tissue for hK3 and skin for hK5) were used.

Two hK3 (PSA) antibodies, PSA8201 and PSA8311 (Medix Biochemica, Finland), both mouse monoclonal antibodies, were used at dilutions of 1:1000 and 1:200, respectively. The PSA antibodies had been tested as described previously (19). An hK5-specific rabbit polyclonal antibody raised against full-length recombinant hK5 protein produced in yeast was used at a dilution of 1:4000. The recombinant hK5 protein was produced and purified by HPLC as described previously (20).

Staining procedures included deparaffinization in xylene for 13 minutes with 2 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 at 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 hK5 primary rabbit polyclonal antibody (Pab) for 1 hour at RT. After 2 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 2 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 (DAB) (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 hK3 and hK5

immunostaining (21, 22). 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 positive and intensity of staining. For the purposes of this study, duct-like 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 inter-examiner variability.

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

RESULTS

hK3 was negative in all tumor tissue examined. The hK5 immunoreactivity was assessed in the cytoplasm of cells that stained positively. In general, all the tumors showed a relatively low overall staining for both ductal and non-ductal cells. The data are summarized in Table I. Normal salivary glands did not express hK5. ACC ductal cells and non-ductal cells, and ANOS non-ductal cells expressed low levels of hK5. In PA, keratinizing epithelia (scored as non-ductal cells) were positively stained for hK5 (Fig. 1). Some hK5 expression was seen in mucoepidermoid carcinomas, in squamous and intermediate type cells (Tab. II). None of these tumors expressed hK5 in significantly raised levels.

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 (10, 11, 23). A number of other kallikreins, such as hK6, hK7, hK8, hK10, hK11, hK13 and hK14, have also been associated with various forms

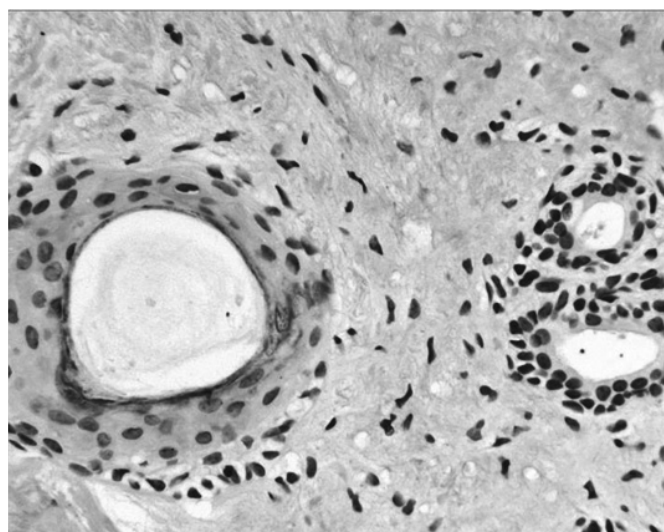


Fig. 1 - hK5 staining (brown staining in cytoplasm of cells) is seen in keratinizing epithelium in pleomorphic adenoma (original magnification x250).

of malignancy (1-4, 6, 24, 25). For example, the KLK8 gene is upregulated in ovarian cancer and its higher expression is associated with a favorable outcome (26, 27). hK5 itself has been associated with ovarian, breast, prostate and testicular cancer (14-17, 20, 28, 29). For these reasons, the possible role of kallikrein family members (hK5 in the current study) in cancer is worth investigating.

In the current study, we did not find any expression of hK3 (PSA) in normal salivary gland tissue nor in salivary gland tumors. Perhaps immunohistochemistry methods alone are not sensitive enough to detect hK3 in salivary gland tissue, although a case of salivary duct carcinoma metastatic to bone and immunohistochemically positive for hK3 has been described in a patient who had markedly elevated serum levels of hK3 (9). We did not examine salivary duct carcinomas in the current investigation. Olsson et al, using in situ hybridization, showed expression of PSA-encoding transcripts in the serous epithelial cells of submandibular gland (12). Expression of both PSA and hK2 was also found in the secretory acini

TABLE I - STAINING IN DUCTAL CELLS AND NON-DUCTAL CELLS AND TOTAL SCORES FOR IMMUNOSTAINING OF SALIVARY TUMORS FOR HUMAN KALLIKREIN 5

Average staining (SD) ¹								
	n ³	Ductal cells			Non-ductal cells			Average of total scores of ductal & non-ductal
		P ⁴	I ⁵	Total	P	I	Total	
Normal	67	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
PA ²	21	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.3)	0.3 (0.9)	0.4 (1.2)	0.2 (0.6)
ACC	27	0.1 (0.4)	0.1 (0.4)	0.1 (0.8)	0.1 (0.6)	0.1 (0.4)	0.2 (1.0)	0.2 (0.9)
PLGA	11	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
ACI	7	Absent	Absent	Absent	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
ANOS	8	Absent	Absent	Absent	0.3 (0.7)	0.1 (0.4)	0.4 (1.1)	0.4 (1.1)

¹For staining score definition, see text. SD, standard deviation

²See non-standard abbreviations

³Number of samples

⁴P, proportion score

⁵I, intensity score

TABLE II - hK5 STAINING IN MUCOEPIDERMOID CARCINOMAS OF MAJOR AND MINOR GLANDS

n = 22	Squamous cells			Mucous cells			Intermediate cells		
	P	I	Total	P	I	Total	P	I	Total
Average	0.4	0.3	0.6	0.0	0.0	0.0	0.5	0.2	0.7
SD	0.8	0.6	1.3	0.0	0.0	0.0	0.9	0.4	1.3

P, proportion score; I, intensity score; SD, standard deviation

and excretory ducts of salivary gland. However, only trace amounts of PSA were also found in approximately half of the tested saliva samples. The low PSA concentrations suggested that PSA is consumed upon secretion from the salivary gland.

Yousef et al, using a sensitive and specific immunofluorometric assay, have reported the expression of hK5 in normal skin at high levels, and in lower levels in breast, salivary gland, esophagus, cerebellum and other tissues, in decreasing order (20). In prostate cancer, the human kallikrein gene 5 (*KLK5*) is significantly downregulated, particularly in stage T3 tumors (29). *KLK5* is highly expressed in normal testis, but lower *KLK5* expression levels are seen in testicular cancers, suggesting an association of higher *KLK5* levels with less aggressive testicular cancer (13, 28).

On the other hand, *KLK5* is a marker of unfavorable prognosis in ovarian cancer (30). hK5 is also found at high levels in ovarian cancer tissue extracts, serum and ascites fluid from ovarian cancer patients (20). It is also a marker of unfavorable prognosis in breast cancer (31), and is elevated in serum in a subset of patients with breast cancer (20).

Michael et al have also reported that hK5 has trypsin-like activity and can digest the extracellular matrix components, collagens type I, II, III and IV, fibronectin, and laminin (15). This suggests that it could play a role in tumor progression, particularly in invasion and angiogenesis, and may represent a novel therapeutic target. Later findings suggest, too, that hK5 can potentially play a role in seminal clot liquefaction and that it may be implicated in prostate cancer progression through growth factor regulation (14). It was described as being part of a proteolytic cascade pathway, implicated in the aforementioned processes.

hK5 protein was previously described as a serine protease with trypsin-like activity, found in the skin (32). It was proposed to have a role in desquamation of the epidermis. It was later also described as being part of a proteolytic cascade in the stratum corneum (33), further emphasizing its role in desquamation. Its presence in keratinizing epithelia is supported by the findings in the current study, in which we observed that hK5 is expressed in the granular layer of keratinizing epithelia of PA.

The results of the current study indicate that salivary gland tumors express low or undetectable levels of hK5. In general, immunoexpression of hK5 varied with individual types of cells and was seen especially in keratin-producing epithelia. This suggests that hK5 is not an important player in many salivary gland tumors, and its expression in PA is related to epithelial turnover rather than tumor growth and development. hK3 appears to play no role in the tumors investigated in this study. Perhaps more sensitive assays, such as immunofluorometric methods, should be used to study the expression of these proteins in salivary gland tumor tissue.

Examination of other kallikreins in salivary gland tumors merits investigation, since almost all kallikreins are expressed in this tissue (1-4). hK5 and hK3, 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, and other glandular structures (4, 5).

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NON-STANDARD ABBREVIATIONS

PA: pleomorphic adenoma
ACC: adenoid cyst carcinoma
PLGA: polymorphous low grade adenocarcinoma
ACI: acinic cell carcinoma
MEC: mucoepidermoid carcinoma
ANOS: adenocarcinoma not otherwise specified
hK: human kallikrein
PSA: prostate-specific antigen
RT: room temperature
PBS: phosphate-buffered saline
Pab: polyclonal antibody
DAB: 3,3'-diaminobenzidine

Address for correspondence:
Dr. Mark Darling
Department of Pathology
University of Western Ontario
1151 Richmond Street
London, Ontario N6A 5C1, Canada
e-mail: mark.darling@schulich.uwo.ca

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