Review

Cellular distribution of human tissue kallikreins: immunohistochemical localization

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Abstract

We have studied the immunohistochemical expression (IE) of eight non-tissue-specific human kallikreins (hKs) (hK5, 6, 7, 10, 11, 12, 13, and 14) in different normal tissues. The IE was always cytoplasmic, showing a characteristic pattern in some tissues. Comparison of the IE of all hKs studied in the different tissues revealed no major differences, suggesting that they share a common mode of regulation. Furthermore, hKs were immunohistochemically revealed in a variety of tissues, indicating that no protein is tissue-specific (except for hK2 and hK3, which have tissue-restricted expression). In general, our results correspond well with data from RT-PCR and ELISA assays. Glandular epithelia constitute the main kallikrein IE sites, and the staining in their secretions confirms that these proteases are secreted. A variety of other tissues express the proteins as well. We have also immunohistochemically evaluated all the above hKs in several malignant tissues. Tumors arising from tissues expressing kallikreins tested positive. Corresponding to the IE in normal glandular tissues, most hKs were expressed in adenocarcinomas. The prognostic value of several hKs was studied in series of prostate, renal cell, colon and urothelial carcinomas.

Keywords: cancer biomarkers; human tissue kallikreins; immunohistochemistry; prognostic markers; serine proteases.

Introduction

Human tissue kallikreins are members of a large multi-gene family of 15 serine proteases (Diamandis et al.,

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Regarding a recommendation for future nomenclature of kallikrein gene-derived proteases, see the article 'A comprehensive nomenclature for serine proteases with homology to tissue kallikreins' by Lundwall et al., this issue pp. 637–641.

2000b; Yousef and Diamandis, 2001). An international group of investigators has agreed on a unified nomenclature, with the genes designated as *KLK1–KLK15* (all located on chromosome 19q13.4) and their encoded proteins as hK1–hK15 (Diamandis et al., 2000a).

Most studies use quantitative RT-PCR to measure the expression of hKs in benign and malignant tissues. The development of monoclonal and polyclonal antibodies against many hKs, and of immunofluorometric ELISAs for quantifying the proteins, has helped in defining their distribution in serum, biological fluids and tissue extracts (Diamandis et al., 2000d; Yousef and Diamandis, 2001, 2002).

In recent reports, many members of the *KLK* gene family have been proposed as new biomarkers for prostate, breast, ovarian, testicular and other cancers. *KLK*s have been found to be differentially expressed in various malignancies (up- or down-regulated) and the decrease or increase in their expression is frequently associated with prognosis or progression. Recent studies suggest that these proteins may be involved in carcinogenesis and tumor metastasis (Diamandis et al., 2000b; Diamandis and Yousef, 2001; Luo et al., 2001, 2002; Yousef and Diamandis, 2001; Chang et al., 2002; Yousef et al., 2002a,d, 2005; Borgono and Diamandis, 2004; Clements et al., 2004).

During the last few years, we have immunohistochemically evaluated most of the above hKs in different normal human tissues and malignancies. Furthermore, in a series of prostate, renal cell, colon and urothelial carcinomas, we have studied their prognostic value (Petraki et al., 2001, 2002a,b, 2003a,b, 2005).

Immunohistochemical study of hKs

The streptavidin-biotin-peroxidase protocol, using the DAKO LSAB+ Peroxidase Kit (DAKO, Mississauga, ON, Canada) was performed on a large number of formalin-fixed and paraffin-embedded tissues from archival, current and autopsy material. Specific polyclonal and several different monoclonal antibodies for eight hKs (hK5, hK6, hK7, hK10, hK11, hK12, hK13, and hK14), raised by immunization with full-length recombinant hKs, were used (dilution 1:500 for polyclonal and 1:150 for monoclonal antibodies). All antibodies for every hK revealed similar immunostaining (IS) patterns in all tissues. Replacement of the primary hK antibody by non-immune rabbit serum and immunoabsorption of the primary hK antibody by mixing it for 1 h with excess recombinant hK before immunostaining resulted in abolition of the IS, suggesting good specificity (Figure 1A–D).
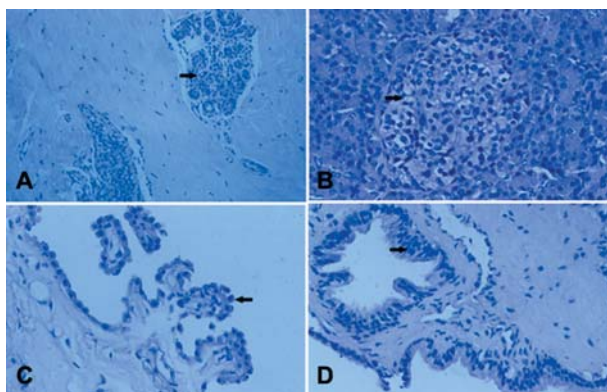
Figure 1   Negative immunostaining controls.
(A) Unstained breast epithelium (arrow) after antibody (hK6 polyclonal antibody) immunoabsorption. (B) Unstained endocrine cells in an islet of Langerhans in the pancreas (arrow) after hK10 polyclonal antibody immunoabsorption. (C) Unstained epithelium of the choroid plexus (arrow) after substituting the primary antibody (hK6 polyclonal) with non-immune rabbit serum. (D) Unstained columnar prostate epithelium (arrow) after substituting the primary antibody (hK10 polyclonal) with non-immune rabbit serum. Original magnification: (B–D) 200×, (A) 100×.

The immunohistochemical expression (IE) was always cytoplasmic, showing a characteristic IS pattern in some tissues: membranous, droplet-like, supranuclear, subnuclear and luminal IE (Petraki et al., 2001, 2002a, 2003a; Yousef and Diamandis, 2002). It is worth mentioning that according to Xi et al. (2004), hK4 appears to be a notable exception, showing predominantly nuclear overexpression in prostate cancer. These preliminary data need to be reproduced, as other studies indicate that this kallikrein, as all other kallikreins, is a secreted protease (Simmer, 2004; Obiezu et al., 2005). In a recent study, Dong et al. (2005) reported that there are two major isoforms of hK4 (KLK4-254/hK4-254 and KLK4-205/hK4-205) expressed in prostate cancer, with different regulatory and expression profiles that imply both secreted and nuclear roles, respectively.

Comparison of the IE patterns of all hKs in different tissues revealed no major differences, suggesting that they share a common mode of regulation (Diamandis et al., 2000a). Furthermore, as all *KLK*s reside on the same chromosomal locus, they share considerable similarities at the gene and protein levels (Petraki et al., 2001, 2002a,b, 2003a,b, 2005; Yousef and Diamandis, 2001; Yousef et al., 2005). It is worth mentioning that our IE results for the hKs studied in different normal human tissues correspond well with relevant data from quantitative methods, mainly RT-PCR and ELISA. According to these studies, apart from *KLK2* and *KLK3*, none of the remaining *KLK*s are tissue-specific, although certain genes are preferentially expressed in some organs (Chu, 1997; McCormack et al., 1995; Rittenhouse et al., 1998; Stenman, 1999; Yousef and Diamandis, 2002; Yousef et al., 2005). Prostate-specific antigen (PSA, hK3) and hK2 are relatively prostatic-specific proteins and have already found important applications as biomarkers for the diagnosis and monitoring of prostate cancer. It is worth mentioning, however, that hK3 and hK2 proteins and mRNA

have been found in significant amounts in the female breast and at lower levels in many other tissues (Rittenhouse et al., 1998; Black and Diamandis, 2000; Black et al., 2000; Yousef et al., 2005). Pancreatic/renal tissue kallikrein (hK1) is one of the most extensively studied members of the kallikrein family. It is known to cleave various prohormones and bioactive peptides, including kininogen, proinsulin, prorenin and procollagenase, and plays a major role in inflammation, heart disease, renal nephritis and diabetic renal disease. The prohormone kininogen is synthesized in the liver and is composed of high-molecular-weight kininogen (120 kDa) and low-molecular-weight kininogen (68 kDa). Lysyl-bradykinin (kallidin) is a decapeptide produced by the proteolytic action of hK1 upon low-molecular-weight kininogen via cleavage between two specific bonds involving Met-Lys and Arg-Ser sequences. As lysyl-bradykinin is a vasoactive peptide that lowers blood pressure, hK1 plays an important role in blood pressure regulation. The actions of lysyl-bradykinin are opposed by angiotensin II, a vasoconstrictive peptide produced from the proteolytic cleavage of angiotensinogen I by angiotensin-converting enzyme (Margolius et al., 1974; Bhoola et al., 1992, 2001; Jaffa et al., 1992; Clements, 1998; Margolius, 1998; Laxmikanthan et al., 2005). hK1 is mainly expressed in salivary glands, pancreas, kidney and pituitary, and it cannot be considered as tissue-specific, as it has also been found in endometrium, ovary and skin (Dietl et al., 1978; ole-MoiYoi et al., 1979; Orstavik et al., 1980, 1981; Pinkus et al., 1983; Terashima et al., 1989; Jones et al., 1990; Vio et al., 1990; Diamandis et al., 2000b; Yousef et al., 2005).

The eight hKs studied (hK5–hK7 and hK10–hK14) were immunohistochemically revealed in a variety of tissues, indicating that none of the proteins is tissue-specific. Immunohistochemistry is a tool superior to other methods, as it defines the protein distribution in different cell types, independently of its quantity in the tissue. This means that a tissue can immunohistochemically express an hK, but can yield negative results using a quantitative method based on whole-cell lysate. This probably explains why we did not find major immunohistochemical differences in tissue expression among the eight hKs, while other methods show some tissue preferences for each hK. It should be mentioned that, similarly, using the more sensitive RT-PCR technique instead of Northern blot analysis, many *KLK* genes are found to be expressed in a wider variety of tissues (Yousef et al., 2001).

Tumors arising in tissues immunohistochemically expressing the eight hKs studied also showed an IE. Glandular epithelia constitute the main hK IE sites, and staining in their secretions suggests that these proteases are secreted (Yousef and Diamandis, 2002). Corresponding to the IE in normal glandular tissues, all hKs studied were expressed in adenocarcinomas, supporting the involvement of kallikreins in the pathogenesis and progression of cancer. A variety of cells of epithelial or other origin located in human organs of the different systems expressed the proteins as well (Yousef at al., 2005).

Table 1 summarizes the cellular distribution of the eight kallikreins studied in different human tissues; a detailed analysis of the IE of all hKs is given below.

Skin and skin appendages

The squamous epithelium of the epidermis was negative for the eight hKs studied. Only the cornified layer showed variable positivity. A strong IE was observed in the secretory and excretory components of the eccrine sweat and apocrine glands (Figure 2A). The keratin-producing cells of the external root sheath of the hair follicles and the sebaceous glands showed weak to moderate expression (Petraki et al., 2001, 2002a, 2003a). Our data support the view that the high concentration of many hKs in skin extracts by ELISA is mostly due to their localization in the appendages and not in the epidermis (Mitsui et al., 2000; Yousef et al., 2000). In two immunohistochemical and *in situ* hybridization studies carried out by Komatsu et al. (2003, 2005), the colocalization of various hKs seemed to be essential for the regulation of serine protease activity in both the skin and the appendages and for steady desquamation and skin barrier function. Furthermore, the increased hK expression in psoriasis vulgaris and atopic dermatitis could be a clue to elucidation of their pathogenesis.

Respiratory system

The epithelium of the upper and lower respiratory tract (nose, paranasal sinuses, larynx, trachea, bronchial tree) and the submucosal glands in these sites expressed the eight hKs (Figure 2B). The alveolar epithelium of the lung parenchyma was negative (Petraki et al., 2001, 2002a, 2003a). In lung cancer, we observed variable hK IE in adenocarcinomas, and generally in non-small cell carcinomas, but not in neuroendocrine small cell carcinomas (unpublished results).

Salivary glands

The epithelium of the excretory ducts of the major and minor salivary glands was consistently positive for the eight hKs studied (Figure 2C). Most mucous and serous alveoli were negative (Petraki et al., 2001, 2002a, 2003a). This IE explains why almost all *KLK*s evaluated by quantitative methods are concentrated in these glands (Orstavik et al., 1980; Diamandis et al., 2000b; Yousef and Diamandis, 2002). As expected, the ductal epithelium of cystadenolymphomas and tumors derived from ductal epithelium expressed all hKs studied (unpublished results). In an immunofluorescence study of submandibular and parotid salivary glands, hK1 was expressed apically in the striated duct cells, whereas it was absent from the main excretory ducts (or present only as a weak luminal rim), acinar cells and cells of the intercalated ducts (Orstavik et al., 1980).

Gastrointestinal system

The non-keratinizing squamous epithelia of the pharynx and esophagus were negative. In contrast, the ductal epithelium of the submucosal glands expressed the eight hKs strongly (Petraki et al., 2001, 2002a, 2003a; see Figure 2D). Our findings support the view that the concentration of several *KLK*s in these organs, based on other techniques, is due to the location (perhaps secretion) of these proteases in the submucosal glands (Kishi et al., 2003). The gastric mucosa expressed hKs focally in all cell types (Figure 2E). All parts of the small and large intestine and the appendix showed IE in all cell types (Petraki et al., 2001, 2002a, 2003a). Adenocarcinomas of the stomach and small and large intestine showed variable IE (Figure 2F). In a recent study, we found that hK10 IE was an independent predictor of unfavorable overall disease-specific survival in colon cancer (unpublished results).

Pancreas, liver, gallbladder and extrahepatic bile ducts

In the exocrine pancreas, IE of the hKs studied was observed in the medium- and small-sized pancreatic ducts, while acinar cells were negative. The epithelium of the bile duct system and the gall bladder was positive. Hepatocytes were negative (Petraki et al., 2001, 2002a, 2003a). Pancreatic adenocarcinomas were variably immunoreactive. In a recent study we found that hK10 was overexpressed in pancreatic cancer, but these findings were of no diagnostic or prognostic value (unpublished results). Strong positivity of the eight hKs studied was found in cells of the islets of Langerhans (Figure 2G). Scattered hK-positive cells were localized in relationship with pancreatic acinar cells. Using a double-immuno-staining method, we documented that all cell types in the islets revealed co-localization of each islet hormone and hK6 and hK10, respectively. Foci of nesidioblastosis and endocrine dysplasia and hormone-producing tumors also revealed strong positivity (Petraki et al., 2002b). There is experimental evidence that kallikrein enzymes present in the islets may be involved in islet hormone processing, particularly in the conversion of proinsulin to insulin. In most reports, the type of tissue kallikrein that sequentially cleaves proinsulin to form the active molecule is specified as hK1 (Yoi et al., 1979; Bhoola et al., 1992; Seidah and Chretien, 1999).

Pancreatic/renal tissue kallikrein (hK1) has been found in pancreatic cells, including the β-cells of pancreatic islets (Dietl et al., 1978; ole-MoiYoi et al., 1979; Orstavik et al., 1980, 1981; Pinkus et al., 1983). It has also been suggested that the increase in kinin and its activation in the acute phase of pancreatitis might be due to pancreatic tissue kallikrein or trypsin originating from the pancreas (Uehara et al., 1989). There are contradictory findings regarding hK1 localization in different parts of the exocrine and endocrine pancreas. In one study using a monospecific antibody against the antigenically identical urinary kallikrein (urokallikrein), immunohistochemical localization of glandular pancreatic kallikrein to the β-cells of the human pancreatic islets was the same as that for insulin in normal human pancreas and in two islet-cell tumors. The authors concluded that the β-cell localization of human pancreatic kallikrein suggests that pancreatic kallikrein may be involved in the physiologic

Table 1   Summary of the immunohistochemical localization of hK5, 6, 7, 10, 11, 12, 13 and 14 in human tissues.

System or organ	Human tissue
Skin	Eccrine sweat and apocrine glands
Respiratory system	Epithelium of the upper respiratory tract
	Epithelium of the bronchial tree
	Submucosal glands
Salivary glands	Ductal epithelium
Gastrointestinal system	Ductal epithelium of the submucosal esophageal glands
	Esophageal cardiac type glands
	Epithelial cells of the stomach, small and large intestine and appendix
Exocrine pancreas	Ductal epithelium
Liver, extrahepatic ducts, gall bladder	Epithelium of the bile duct system
	Epithelium of the gallbladder
Urinary system	Epithelium of the urinary tubuli of the kidney
	Umbrella cells of the urothelium
Male reproductive system	Secretory cells of the prostate
	Epithelium of the ejaculatory ducts and the seminal vesicles
	Spermatic epithelium and Leydig cells in the testis
	Epithelium of the rete testis, epididymis and ductus deferens
	Columnar epithelium of the urethra
	Littre's and Cowper's glands
Female reproductive system	Epithelium of the breast ductal and lobuloalveolar structures
	Endometrium
	Endocervical epithelium and glands
	Surface epithelium of the ovary, luteinized stromal cells
Endocrine organs	Endocrine cells of the islets of Langerhans in pancreas
	Medulla of the adrenal glands
	Follicular cells of the thyroid
	Oxyphilic cells of the parathyroid glands
	Lactotrophs and corticotrophs in the anterior pituitary gland
Central and peripheral nervous system	Choroid plexus
	Neurons and glial cells in the cerebral cortex
	Nerves and ganglia
Lymphatic organs	Submucosal glands of the tonsils
	Hassall's corpuscles of the thymus
Mesothelium	Pleural, pericardial and peritoneal mesothelium
Mesenchymal tissues	Chondrocytes, endothelium

activation of proinsulin (ole-MoiYoi et al., 1979; Pinkus et al., 1983). Using anti-urinary and anti-pancreatic kallikrein sera, they observed moderate acinar and ductal immunostaining in the absence of pretreatment of the tissue with trypsin or pronase. Short incubation with either enzyme permitted the discrete localization of islet β-cell kallikrein antigen, while increased pronase concentrations decreased kallikrein antigen in both islets and exocrine tissue and led to islet destruction. The authors concluded that both antibody specificity and tissue preparation influence kallikrein localization in human pancreas. The studies of Orstavik et al. (1980, 1981) strongly indicate that glandular pancreatic kallikrein is predominantly located in acinar cells of the exocrine pancreas. Dietl et al. (1978) also reported localized pancreatic tissue kallikrein in the acinar cells of the pancreas, but not in the islets of Langerhans or in the interlobular ducts. Chao et al. (1980) reported that it is unlikely that pancreatic tissue kallikrein is involved in the *in vivo* conversion of proinsulin to insulin, as appreciable quantities of the enzyme were not detected in pancreatic islets by direct radioimmunoassay and bioassay.

Urinary system

The epithelium of the urinary tubuli of the kidney showed IE for the eight hKs, while glomeruli were negative (Petra-

ki et al., 2001, 2002a, 2003a; see Figure 2H). These hKs were variably expressed in renal cell carcinoma, mostly in sarcomatoid, oncocytic and papillary subtypes (Figure 2I). In a recent study carried out by our group, the IE of hK5, hK6, hK10 and hK11 in renal cell carcinoma was decreased, although these hKs seemed to be an unfavorable prognostic factor (Petraki et al., 2005). In the study of Cumming et al. (1994), the dominant site of hK1 immunoreactivity was the distal tubule. Several studies indicate that the renal kallikrein-kinin system is involved in renal pathophysiology. Naicker et al. (1997) observed additional IE of renal tissue kallikrein in proximal tubule cells in women with pre-eclampsia, while in healthy controls the staining was confined to distal connecting tubules and the collecting ducts. In another study, Naicker et al. (1999) found decreased renal tissue kallikrein and kinin B2 receptors, and increased kinin B1 receptor immunoexpression in the kidneys of patients with renal disease in comparison with control kidneys. Ramsaroop et al. (1997) observed a reduction in the intensity of kallikrein immunostaining in renal transplant tissue, but maintenance of the localization in distal connecting tubules and the collecting ducts, as in normal controls. Rae et al. (1999) found that *KLK1–3* and a novel *KLK1* mRNA transcript were expressed in a renal cell carcinoma cDNA library.

In the urothelium covering the entire urinary tract, superficial umbrella cells and only some scattered inter-
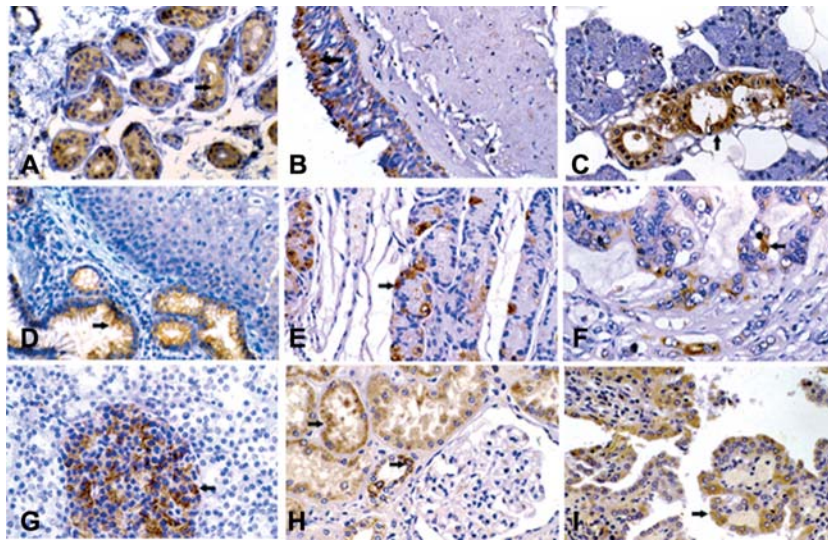
Figure 2   Immunohistochemical expression analysis of hK5, hK6, hK7, hK10, hK11 and hK13.
(A) Expression of hK7 by the epithelium of eccrine glands of the skin (arrow, monoclonal antibody clone 73.2). (B) Expression of hK13 by the columnar epithelium of the bronchus (arrow, monoclonal antibody clone IIC1). (C) Expression of hK5 by the ductal epithelium of the parotid gland (arrow, polyclonal antibody). (D) Expression of hK7 by the epithelium of the esophageal glands (arrow, monoclonal antibody clone 73.2). (E) Expression of hK13 by the epithelium of the gastric mucosa of the antrum (arrow, monoclonal antibody clone 2–17). (F) Expression of hK6 by a colon adenocarcinoma (arrow, polyclonal antibody). (G) Expression of hK10 by the endocrine cells in an islet of Langerhans in the pancreas (arrow, monoclonal antibody clone 5D3). (H) Expression of hK11 by the epithelium of the proximal and distal convoluted urinary tubuli in the kidney (arrows, monoclonal antibody). (I) Expression of hK11 in a papillary renal cell carcinoma (arrow, monoclonal antibody). All original magnifications 200×. Dilutions were 1:500 for all polyclonal antibodies and 1:150 for all monoclonal antibodies.

mediate urothelial cells expressed the eight hKs (Petraki et al., 2001, 2002a, 2003a). The IE in urothelial carcinomas was variable, with strong perinuclear staining in some cases (Figure 3A). In a series of urothelial carcinomas of the urinary bladder, the IE pattern for hK5, 6, 10 and 11 revealed full-thickness staining in the papillary areas and diffuse staining in the invasive areas. Residual superficial umbrella cells were positive. None of these hKs seemed to correlate with grade and pathological stage. Foci of urothelial dysplasia and urothelial carcinoma *in situ* mostly showed a full-thickness IE. Our findings suggest that these hKs could be markers of
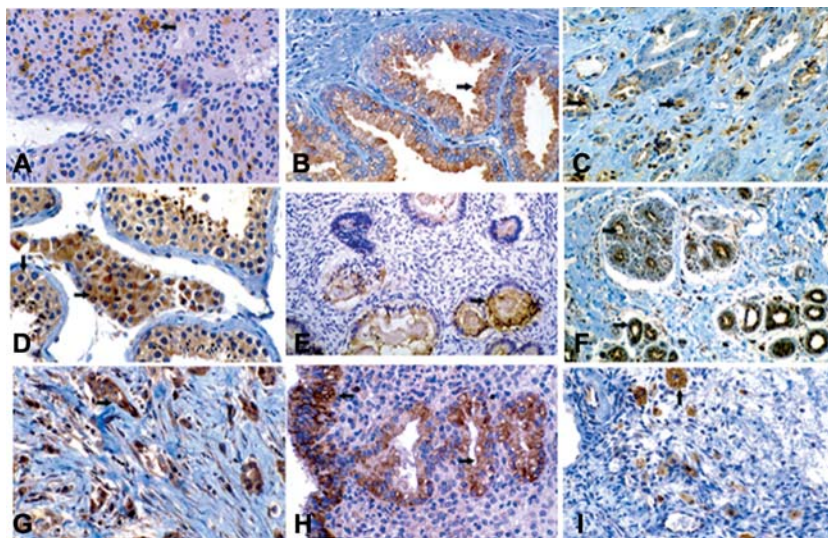

Figure 3   Immunohistochemical expression analysis of hK10, hK11, hK13 and hK14.
(A) hK10 expression in a low-grade urothelial carcinoma of the urinary bladder (arrow, monoclonal antibody clone 5D3, magnification 200×). (B) hK11 expression in secretory epithelium of the prostate gland (arrow, polyclonal antibody, magnification 200×). (C) hK11 expression by a Gleason score 6 prostate carcinoma (arrows, polyclonal antibody, magnification 100×). (D) hK14 expression by spermatic epithelium and stromal Leydig cells in the testis (arrows, polyclonal antibody, magnification 200×). (E) hK10 expression by epithelial elements in a testicular immature teratoma (arrow, monoclonal antibody clone 5D3, magnification 100×). (F) hK14 expression by epithelium of lobuloalveolar structures of the breast (arrows, polyclonal antibody, magnification 100×). (G) hK14 expression in a ductal breast carcinoma, grade II (arrow, polyclonal antibody, magnification 100×). (H) hK13 expression by glandular epithelium of the endometrium (arrows, polyclonal antibody, magnification 200×). (I) hK14 expression by luteinized stromal cells of the ovary (arrow, polyclonal antibody, magnification 100×). Dilutions were 1:500 for all polyclonal antibodies and 1:150 for all monoclonal antibodies.

urothelial differentiation and may play a significant role in urothelial carcinogenesis (unpublished results).

Male reproductive system

In benign prostatic epithelium, the luminal secretory cells were stained with the eight hK antibodies (Figure 3B). However, mainly membranous staining was occasionally prominent in the basal cells and in foci of basal cell hyperplasia (Petraki et al., 2001, 2002a, 2003a). A possible explanation is the fact that both cell types derive from the same stem cell and can have similar phenotypes in several pathological benign or malignant conditions. Generally, all hKs studied had the same IS pattern in the benign prostate gland as for hK2 and hK3 (Pretlow et al., 1991; Obiezu et al., 2002). In prostate cancer, the eight hKs showed variable IE (Figure 3C). Prostatic intraepithelial neoplasia showed mostly luminal apical IS. In a recent immunohistochemical study we found that high-grade prostatic cancers expressed hK6, hK10 and hK13 at a higher percentage than low grade cancers, though all three hKs were down-regulated in cancer (Petraki et al., 2003b). Studies carried out by our group and others, based mostly on RT-PCR, have shown variable prognostic significance of several *KLK* genes in prostate cancer (Abrahamsson et al., 1988; Gallee et al., 1990; Pretlow et al., 1991; Clements and Mukhtar, 1997; Darson et al., 1997, 1999; Tremblay et al., 1997; Siivola et al., 2000; Diamandis et al., 2002; Obiezu et al., 2002; Yousef et al., 2002c; Stephan et al., 2003). The epithelium of the ejaculatory ducts, seminal vesicles, ductus deferens, epididymis and the efferent ductules revealed positivity for the eight hKs as well. Spermatic epithelium in the testis showed a variable IE, with spermatogonia mostly positive. Leydig cells were strongly positive. The columnar epithelium of the penile urethra, as well as Littre's and Cowper's glands, expressed these hKs (Petraki et al., 2001, 2002a, 2003a; see Figure 3D). An immunohistochemical study of all hKs examined in testicular germ cell tumors revealed weak positivity in seminomas and stronger positivity in embryonal carcinomas and teratomas (Figure 3E). Weak IS was sometimes observed in intratubular neoplasia (unpublished results). Generally, in testicular germ cell tumors, some *KLK* genes, including *KLK5*, *KLK10*, *KLK13* and *KLK14*, are found to be significantly down-regulated (Chang et al., 2001; Yousef et al., 2002b; Luo et al., 2003).

Female reproductive system

IE of the eight hKs studied was identified in ductal and lobuloalveolar structures of the non-malignant breast (Figure 3F). Luminal secretions were also positive. Foci of apocrine metaplasia, apocrine cysts and epithelial ductal hyperplasia showed strong staining. In infiltrating and *in situ* breast carcinoma of both ductal and lobular type, focal IE of these hKs was observed (Petraki et al., 2001, 2002a, 2003a; see Figure 3G). As assessed by quantitative RT-PCR, *KLK*s mostly seem to be favorable prognostic markers for breast carcinoma (Chang et al.,

2002; Luo et al., 2002; Yousef et al., 2002a,d,e, 2003d; Junes et al., 2003).

KLK1, *KLK2* and *KLK3* have been detected in human endometrium, confirming the presence of a local kallikrein-kinin system in this tissue (Clements and Mukhtar, 1994). In another RT-PCR and Southern blot study, Clements et al. (1994) showed that *KLK1* was expressed in human endometrium and myometrium. Using immunohistochemistry, hK1 was localized mainly in the glandular epithelium of the endometrium. Furthermore, *KLK1* gene expression was increased during the proliferative phase of the menstrual cycle, suggesting a role for this kallikrein in the preparation of the endometrium for implantation. *KLK1* was also expressed in a range of endometrial cancers, although at lower levels than that observed for normal human endometrial tissues (Clements and Mukhtar, 1997). In our studies, the endometrium expressed the eight hKs studied in both proliferative and secretory phases (Figure 3H), with characteristic IE in areas of decidual change. In the placenta, hKs were localized in the endothelia, calcifications of the villi, and 'X' cells, and focally in trophoblastic cells (Petraki et al., 2001, 2002a, 2003a). Focal IE was also observed in adenocarcinomas of the endometrium (unpublished results). IE of the eight hKs was observed in the mucin-secreting epithelium of the endocervix and the tubular cervical glands. Squamous epithelium of the exocervix and the vagina was negative. Cane et al. (2004) observed hK8 IE in cervical cancer, but no expression in normal cervical keratinocytes.

A clear immunoreaction was observed in premordial follicles, granulosa lutein cells in the corpora luteum and sparse luteinized cells in the stroma of the ovary (Figure 3I). Surface epithelium of the ovary expressed all hKs as well (Petraki et al., 2001, 2002a, 2003a; see Figure 4A). Epithelial serous and mucinous ovarian tumors, both benign and malignant, expressed a high percentage of all hKs (Figure 4B), a finding that is in accord with previous observations. Data from other groups and our laboratory indicate that *KLK* genes are differentially expressed in ovarian cancer and may have a favorable or unfavorable prognostic value (Tanimoto et al., 1999; Underwood et al., 1999; Dong et al., 2001, 2003; Kim et al., 2001; Luo et al., 2001; Obiezu et al., 2001; Hoffman et al., 2002; Shvartsman et al., 2003; Yousef et al., 2003b,c; Shigemasa et al., 2004; Davidson et al., 2005).

Endocrine organs (adrenal glands, thyroid gland, parathyroid glands, pituitary gland)

Moderate positivity of all hKs studied was observed in the adrenal medulla. Focal IE was revealed in follicular cells in the thyroid gland, mainly in hyperplastic conditions and in oxyphilic cell metaplasia (Petraki et al., 2001, 2002a, 2003a; see Figure 4C). These findings are in accord with the demonstration of hK2 and hK3 in oxyphilic cells of the thyroid, suggesting another similarity among hKs (Magklara et al., 2000). In thyroid cancer, focal IE of the hKs studied was frequently observed in papillary and anaplastic carcinomas (Figure 4D). Strong IE was noted for oxyphilic cells of the parathyroid glands.
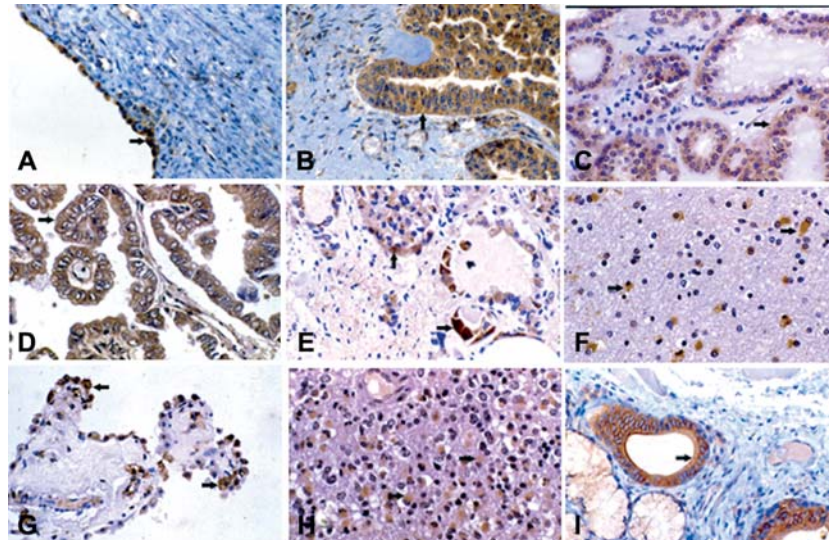
Figure 4 Immunohistochemical expression of hK5, hK6, hK7, hK10, hK13 and hK14.
(A) hK14 expression in ovarian surface epithelium (arrow, polyclonal antibody). (B) hK14 expression in a moderate cystadenocarcinoma of the ovary (arrow, polyclonal antibody). (C) hK10 expression in epithelium of hyperplastic follicles of the thyroid gland (arrow, monoclonal antibody, clone 5D3). (D) hK6 expression by a papillary thyroid carcinoma (arrow, polyclonal antibody). (E) hK13 expression by endocrine cells in the anterior lobe of the pituitary gland (arrows, monoclonal antibody clone 2-17). (F) hK10 expression by glial cells in the brain (arrows, monoclonal antibody clone 5D3). (G) hK13 expression in the choroid plexus epithelium (arrows, monoclonal antibody clone 2-17). (H) hK5 expression by a glioma (arrows, monoclonal antibody clone 6.10). (I) hK7 expression by ductal epithelium of the submucosal glands of the tonsils (arrow, monoclonal antibody clone 85.2). All original magnifications 200×, except (A) and (G) (100×). Dilutions were 1:500 for all polyclonal antibodies and 1:150 for all monoclonal antibodies.

Chief cells remained mostly unstained. In the anterior lobe of the adenohypophysis, lactotrophs and cortico-trophs expressed these proteins strongly. Characteristic strong positivity was also shown in prolactin immuno-reactive epithelium-lined follicular and ductal formations in the poorly developed pars intermedia (Figure 4E). Pro-lactinomas were also positive. Pituitocytes of the pars nervosa were negative (Petraki et al., 2001, 2002a, 2003a). It is worth mentioning that pancreatic hK1 has been found in lactotrophs of the rat and human anterior pituitary (Jones et al., 1990; Vio et al., 1990). In a Northern blot and RT-PCR study, Clements et al. (1996) demonstrated a low level of expression of *KLK1*, as well as *KLK2* and *KLK3*, in the human pituitary.

Central and peripheral nervous system

Gray- and white-matter cerebral cortex neurons, as well as glial cells (astrocytes and oligodendrocytes), were weakly to moderately immunoreactive for the eight hKs studied (Figure 4F). The most striking finding was the strong positivity of these hKs in the epithelium of the choroid plexus, which is responsible for the production of cerebrospinal fluid (Petraki et al., 2001, 2002a, 2003a; see Figure 4G). Several studies have shown that a number of *KLK* genes, including *KLK1*, are expressed in the central nervous system and may have a potential role, such as in the diagnosis and monitoring of Alzheimer's disease (Little et al., 1997; Yamanaka et al., 1999; Dia-mandis et al., 2000b,c; Mitsui et al., 2000, 2002; Petraki et al., 2001, 2002a, 2003a; Shimizu-Okabe et al., 2001; Yousef and Diamandis, 2001; Yousef et al., 2003a). Little et al. (1997) detected positive hK6 IS in monkey cortex

cells lining the perimeter of cortical microvessels, in human brains of patients with Alzheimer's disease, and in microglial cells, indicating a role of this protease in brain disease. Terayama et al. (2004) observed *KLK6* and *KLK8* expression (in mRNA and at the protein level) in oligodendrocytes, with differential distribution after injury to the spinal cord. In a small number of brain tumors (gliomas and oligodendrogliomas), we observed focal IE of the eight hKs studied (unpublished results) (Figure 4H).

In the peripheral nervous system, all types of nerves and ganglia showed IE for all hKs (Petraki et al., 2001, 2002a, 2003a).

Lymphatic organs

In general, the lymphatic organs did not express any of the hKs studied. Characteristic positivity was only observed in Hassall's corpuscles of the thymus. In ton-sils, these hKs were expressed by the submucosal glands, mainly by the ductal portion (Figure 4I). Accord-ing to our immunohistochemical findings, the high levels of several hKs measured in tonsils by quantitative meth-ods represent the localization of these proteases in the glands and not in the lymphatic tissue. Inflammatory cells, mainly polymorphonuclear leukocytes, expressed the hKs studied in different tissues (Diamandis et al., 2000b; Petraki et al., 2001, 2002a, 2003a; Yousef and Diamandis, 2001). Polymorphonuclear leukocytes from synovial fluid of patients with rheumatoid arthritis showed reduced tissue kallikrein and kinin immunoreactivity in comparison with blood polymorphonuclear leukocytes from the same patients and healthy subjects. These results support the hypothesis that tissue kallikrein

released from the granules of rheumatoid arthritis synovial fluid polymorphonuclear leukocytes cleaves the kinin moiety from multifunctional kininogen protein on the surface of these cells (Williams et al., 1997).

Mesothelium

The pleural, pericardial and peritoneal mesothelium was immunoreactive for the eight hKs studied, especially in hyperplastic conditions (Petraki et al., 2001, 2002a, 2003a).

Mesenchymal tissues

In general, none of the eight hKs was immunohistochemically expressed in mesenchymal tissues. Only chondrocytes, endothelia and the wall of small vessels expressed these hKs in some cases, a finding also referred to in other studies (Petraki et al., 2001, 2002a, 2003a). It has been suggested that hKs are synthesized and secreted by human vascular endothelial cells (Wolf et al., 1999; Yayama et al., 2003). Using RT-PCR and Southern blotting techniques, KLK1 mRNA was detected in human umbilical vein endothelial cells (Dedio et al., 2001). The above-mentioned studies suggest that endothelial cells express hK1, which may serve in the local generation of vasoactive kinins.

Conclusions and future directions

All hKs are immunohistochemically expressed in a variety of tissues, indicating that no protein, except hK2 and hK3, is tissue-specific. The cytoplasmic expression of all hKs, mainly in glandular epithelia and their secretions, suggests that these proteases are secreted. Most hKs are expressed in adenocarcinomas, as well as in tumors arising from tissues expressing these proteins. The data from our studies on prostate, renal cell, colon and urothelial carcinomas support the view that hKs may be involved in the progression of these malignancies.

In the future, we will continue these immunohistochemical studies with normal and malignant tissues to further evaluate the clinical significance of hKs as prognostic markers.

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