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The Spectrum of Human Kallikrein 6 (Zyme/Protease M/Neurosin) Expression in Human Tissues as Assessed by Immunohistochemistry

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SUMMARY The KLK6 gene is a new member of the human kallikrein gene family and encodes for a secreted protease, human kallikrein 6 (hK6; also known as zyme/protease M/ neurosin). No study has as yet reported detailed immunohistochemical localization of hK6 in human tissues. Our purpose was to examine the expression of hK6 in human tissues by immunohistochemistry. We have analyzed 199 paraffin blocks from archival, current, and autopsy material prepared from almost every normal human tissue. We employed an hK6specific polyclonal rabbit antibody and avidin-biotin to localize hK6 by IHC. The staining pattern, the distribution of the immunostaining, and its intensity were studied in detail. The IHC expression of zyme was generally cytoplasmic. Various normal human tissues expressed the protein abundantly. Glandular epithelia constituted the main immunoexpression sites, with representative organs being the breast, prostate, kidney, endometrium, colon, appendix, salivary glands, bile ducts, and gallbladder. The small intestine, stomach, endocervix, Fallopian tube, epididymis, bronchus, and upper respiratory tract showed a focal expression as well. Choroid plexus epithelium, peripheral nerves, and some neuroendocrine cells (including the islets of Langerhans, cells in the anterior pituitary gland, and adrenal medulla) expressed the protein strongly and diffusely. A characteristic immunostaining was observed in the Hassall's corpuscles of the thymus, the oxyphilic cells of the thyroid and parathyroid glands, the primordial follicles of the ovary, dendritic cells mainly in the spleen, and in various cells of the placenta. (J Histochem Cytochem 49:1431–1441, 2001)

KEY WORDS

human kallikrein 6 immunohistochemical expression normal human tissues secreted proteins serine proteases cancer biomarkers

UNTIL RECENTLY, the human kallikrein gene family was believed to consist of only three genes: pancreatic/ renal kallikrein (KLK1, encoding for hK1 protein), human glandular kallikrein 2 (KLK2, encoding for hK2 protein), and human kallikrein 3 (KLK3, encoding for hK3 protein or prostate-specific antigen, PSA). The latter two kallikreins, PSA and hK2, are relatively prostate-specific and have already found important applications as biomarkers for the diagnosis and monitoring of prostate cancer (Chu 1997; Rittenhouse et al. 1998; Diamandis 1999; McCormack et al. 1995; Stenman 1999; Diamandis et al. 2000a).

New members of the human kallikrein gene family have recently been discovered (Diamandis et al. 2000a). This gene family now consists of 15 genes which all encode for trypsin-like or chymotrypsin-like serine proteases, show significant homology at both the DNA and amino acid level, and are localized at the chromosomal locus 19q13.4. This area of investigation has recently been reviewed (Diamandis et al. 2000a; Yousef and Diamandis 2001).

The KLK6 gene (encoding for human kallikrein 6, hK6) has been cloned independently by three groups of investigators and was previously given the names

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zyme (Little et al. 1997) (cloned from brain tissue), protease M (Anisowicz et al. 1996) (cloned from breast tissue), and neurosin (Yamashiro et al. 1997) (cloned from a colon carcinoma cell line). Recently, uniform nomenclature for all newly discovered and traditional kallikrein genes has been established (Diamandis et al. 2000b). The KLK6 gene encodes for a trypsin-like serine protease of 244 amino acids, of which 16 amino acids constitute the signal peptide and five amino acids the activation peptide. The mature enzyme consists of 223 amino acids. It has been previously predicted that hK6 is a secreted protein (Anisowicz et al. 1996; Little et al. 1997; Yamashiro et al. 1997; Yousef et al. 1999). This was recently verified by the finding of hK6 protein in various biological fluids, including cerebrospinal fluid, breast milk, nipple aspirate fluid, breast cyst fluid, male and female serum, seminal plasma, amniotic fluid and breast cancer cytosols (Diamandis et al. 2000c). Little et al. (1997) suggested that this enzyme has amyloidogenic potential in the brain and may play a role in the development and progression of Alzheimer's disease. Anisowicz et al. (1996) have cloned the same gene by the method of differential display, and found that it is downregulated in aggressive forms of breast cancer. The same gene was cloned by Yamashiro et al. (1997) from the human colon adenocarcinoma cell line COLO 201.

Recently, we have established the genomic organization and the hormonal regulation of the KLK6 gene and studied its tissue expression by rRT-PCR (Yousef et al. 1999). We found that this gene is abundantly expressed in many tissues, including the salivary gland, brain, uterus, heart, thymus, prostate, liver, breast, thyroid, spleen, placenta, trachea, testis, and kidney (Yousef et al. 1999). We have further raised polyclonal mouse and rabbit antibodies and developed an immunofluorometric procedure for measuring hK6 with high sensitivity and specificity (Diamandis et al. 2000c). Using this assay, we have recently found that hK6 is elevated in serum of ovarian cancer patients and in serum and cerebrospinal fluid of patients with Alzheimer's disease (Diamandis et al. 2000d; Diamandis et al. 2000e). However, no study has as yet described the immunolocalization of hK6 in human tissues. Here we describe immunohistochemical localization of hK6 in diverse human tissues.

Materials and Methods

This was an IHC study on almost all normal human tissues to establish the expression of hK6. Parts of an organ with different histology (e.g., stomach, fundus, body, antrum) were examined separately. A paraffin block of three different cases for every tissue (organ, all parts with different histology) was selected. Cases with malignancy in adjacent sites of the organ were excluded to avoid phenotypic changes that may be associated with cancer. Tissues that exist in several organs (e.g., fat, muscle, vessels, peripheral nerves, ganglia, and neuroendocrine cells) were not studied separately. A total of 199 paraffin blocks were examined. A total of 165 blocks were from archival or current material from 132 cases and the rest were autopsy material from two cases (Table 1).

The avidin-biotin complex IHC method was performed on 4-µm-thick sections using two hK6-specific antibodies, one rabbit polyclonal and one mouse monoclonal. Both antibodies were raised in-house against full-length hK6 produced recombinantly in a mammalian stable cell line system (Little et al. 1997). The recombinant hK6 protein was purified by HPLC as previously described (Little et al. 1997). The specificity of the antibodies was evaluated during development of the immunofluorometric assay, as previously described, and by Western blotting analysis (Diamandis et al. 2000c; and our unpublished data). We found no detectable crossreactivity from other closely related antigens such as hK2, hK3, and hK10 (Diamandis et al. 2000c). All tissues were fixed in buffered formalin. Staining procedures included deparaffinization in xylene and then two changes of xylene at room temperature, followed by rehydration by transfer through graded alcohols. Endogenous peroxidase activity was blocked with 0.5% H₂O₂ in methanol for 10 min. The sections were pretreated with citrate buffer (pH 6.1) in a microwave for 5 min and incubated overnight with the hK6 primary rabbit polyclonal antibody (1:500) and the hK6 primary mouse monoclonal antibody (1:150) in 3% BSA. After washing with Tris-buffered saline (TBS, pH 7.6) for 15 min, they were incubated with a biotinylated secondary antibody for 10 min. After washing with TBS, we incubated with the avidin-peroxidase complex for 10 min. The next steps included application of diaminobenzidine (DAB) substrate and incubation for 10 min, washing in running water, staining with hematoxylin for 1 min, washing in running water again, dehydration through graded alcohols, and covering by DPX. A negative control section was always included in which the primary antibody was substituted with non-immune rabbit or mouse serum. In addition, for selected tissues we performed immunoabsorbtion of the primary hK6 antibody by mixing it for 1 hr with excess recombinant hK6 before immunostaining.

The staining pattern, the distribution of the immunostaining in each tissue, and the intensity of the staining were studied in detail.

Results

The hK6 immunoreactivity using the polyclonal and the monoclonal antibody was generally localized in the cytoplasm. Both antibodies revealed the same distribution of the antigen in all tissues. Replacement of the primary antibody with non-immune serum or immunoabsorbtion of the primary antibody abolished the immunostaining in all tissues examined. These findings indicate that our staining procedure is specific for hK6. The protein was revealed in a variety of tissues, indicating that this is not a tissue-specific pro-

Table I insues studied for the immunohistochemical expression of numan kallikreir	Table 1	Tissues studied for th	e immunohistochemical	l expression of human	kallikrein 6
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Organ	Special tissue	No. of cases (134)	No. of paraffin blocks (199)
Brain and pituitary gland	Cortex, medulla, cerebellum, choroid plexus, meninges, pituitary	2 (autopsy)	34
Spinal cord		3	3
Heart		3	3
Lymph nodes		3	3
Spleen		3	3
Bone marrow		3	3
Thymus		3	3
Tonsils		3	3
Thyroid gland		3	3
Parathyroid gland		3	3
Pituitary gland		3	3
Adrenal gland	Cortex, medulla	3	3
Skin		3	3
Mouth mucosa		3	3
Paranasal sinuses		3	3
Rhinopharynx		3	3
Esophagus		3	3
Stomach	Fundus, body, antrum	3	9
Small intestine	Duodenum, ieiunum, ileum	3	9
Large intestine	Cecum, ascending colon, transverse colon, descending colon, sigmoid, rectum	3	18
Anus		3	3
Appendix		3	3
Larvnx		3	3
Bronchus		3	3
		3	3
Major salivary glands		3	3
Liver		3	3
Gallbladder		3	3
Pancreas		3	3
Kidney	Cortex medulla	3	6
Urinary bladder		3	3
Prostate gland		2	2
		2	2
Cavernous bodies		2	2
Tostic		2	2
Poto tostic		2	2
		с С	2
Ejaculatory ducts		3	3
Epididymis		3	3
Ovary		3	3
Uterus	Corpus, cervix	3	6
vagina		3	3
vuiva		3	3
Fallopian tube		3	3
Placenta		3	3
Breast		3	3

tein. hK6 is mainly expressed by glandular tissues, but our data suggested that it could also serve as a neuroendocrine marker. The distribution and the expression levels of hK6 in various tissues are described below and further summarized in Table 2.

Central and Peripheral Nervous System

Strong and diffuse positivity in the epithelium of the choroid plexus was observed. (Figures 1A and 1B). In the cerebellum, the antigen was expressed weakly in

the Purkinje cells and the stellate (basket cells), whereas the granular cells were negative. In the entire central nervous system the nerve cells showed weak immunostaining as well (Figure 1C). Glial cells showed a weaker expression focally. Staining of the peripheral nerves was intense (Figure 1D).

Female Reproductive System

The epithelium of the breast stained positive. Cytoplasmic and brush-border distribution was observed,

Table 2Tissues with a hK6 immunoexpression

Tissue	Immunostaining characteristics ^a
Choroid plexus	D
Glial cells	D, weak
Peripheral nerves	D
Breast	C, BB, LS
Endometrium	C (droplets), BB, LS
Endocervix	PN, BB
Fallopian tube	SpN, BB
Ovary	Premordial follicles
Placenta	Endothelia, X-cells
Prostate	D
Epididymis, seminal vesicles, spermatic cord	SpN, BB
Kidney (urinary tubuli)	с
Colon, appendix	SbN (colon), SpN (appendix)
Small intestine	Focal C
Stomach	C, SbN, BB, parietal cells
Bile ducts, gallbladder	C, BB
Pancreas	Islets of Langerhans
Bronchus, larynx, trachea,	BB, C (ducts)
rhinopharynx, paranasal sinuses	
Salivary glands	C
Spleen	Dendritic cells
Thymus	Hassall's corpuscles
Adrenal gland (medulla)	C
Thyroid gland	Focal, mainly in hyperplasia
Parathyroids	Oxyphilic cells
Diffuse neuroendocrine system	D

^aD, diffuse; C, cytoplasmic; BB, brush border; LS, luminal secretions; PN, paranuclear; SpN, supranuclear; SbN, subnuclear.

and luminal secretions were positive as well (Figures 2A–2D). A similar immunophenotype appeared in the columnar epithelium of the endometrium and the endocervix of the uterus. A characteristic droplet-like, widely distributed expression was noted in the endometrium (Figure 3A), but staining in the endocervix was focal and paranuclear. No major differences were seen during the menstrual cycle. The myometrium was negative. Weak and focal expression by the squamous cervical epithelium could not be safely considered as positive (see Discussion). The squamous epithelium of the vagina was negative. Supranuclear cytoplasmic, brush-border, and ciliary staining was revealed in the Fallopian tubes. Characteristic was the positivity in the primordial follicles of the ovary. In the placenta the protein was localized in the endothelia, in calcifications of the villi, as well as in "X" cells and, focally, in trophoblastic cells.

Genitourinary Tract

Strong diffuse immunostaining was observed in the prostate columnar cells. Basal cells remained unstained. Because we did not use a double-staining method, it was not possible to evaluate the positivity of the neuroendocrine cells. In general, hK6 had the same immunostaining pattern in the benign prostate gland as hK2 and hK3 (Figures 3B–3D). A focal, mainly suprabasal cytoplasmic, brush-border and ciliary staining in the epithelium of the epididymis, seminal vesicles, and spermatic duct was the rule. The urinary tubuli of the kidney (Figure 4A) showed immunoexpression as well. It is doubtful if weak focal expression by the umbrella cells of the urothelium and the Leydig cells of the testis could be considered as positive. Cavernous bodies were negative.

Gastrointestinal Tract

The glandular epithelium of the large bowel and the appendix showed strong, diffuse cytoplasmic, mainly subnuclear (large intestine) and supranuclear (appendix) immunostaining in the enterocytes (Figure 4B). No clear differences were noted among the different segments of the colon. Expression in the small intestine was focal cytoplasmic, mainly subnuclear in the duodenum and mainly peri- and supranuclear in the ileum. The antrum of the stomach showed a focal cytoplasmic, mainly subnuclear and brush-border staining; the body showed a brush-border and parietal cell expression (Figure 4C). Strong positivity in foci of intestinal metaplasia in the gastric mucosa was the rule (Figure 4D). The duct epithelium of the eosophageal glands expressed the antigen as well. Expression by neuroendocrine cells throughout the gastrointestinal tract was generally obvious. The reactivity by basal cells in the epithelium of the esophagus and the anus was considered to arise from neuroendocrine cells. We found strong positivity in the cells of the islets of Langerhans in the pancreas (Figures 5A-5D). The acinar cells of the exocrine pancreas were negative. Only some scattered positive cells were observed between them. The epithelium of the medium-sized pancreatic ducts showed a cytoplasmic and mainly brush-border immunostaining. Hepatocytes were negative. A cytoplasmic and brush border immunostaining was observed in the bile ducts and the gallbladder mucosa (Figure 6A).

Figure 1 (A) Strong hK6 immunostaining in the choroid plexus epithelium (polyclonal antibody). (B) Strong hK6 immunostaining in the choroid plexus epithelium (monoclonal antibody). (C) hK6 immunoexpression in the nerve cells of the brain (monoclonal antibody). (D) Intense immunoexpression of hK6 in the peripheral nerves (polyclonal antibody). Original magnification ×200.

Figure 2 (A) Cytoplasmic immunostaining for hK6 in breast epithelium (polyclonal antibody). (B) Cytoplasmic immunostaining for hK6 in breast epithelium (monoclonal antibody). (C) Unstained breast epithelium after antibody immunoabsorption. (D) Unstained breast epithelium with non-immune rabbit serum. Original magnifications \times 200.





Respiratory Tract

A mainly brush-border staining in the respiratory epithelium of the bronchus, larynx, trachea, rhinopharynx, and paranasal sinuses was observed (Figure 6B). The staining in the ducts was cytoplasmic. The alveoli of the lung were negative.

Salivary Glands (Major and Minor) and Skin Appendages

Cytoplasmic positivity in the ductal epithelium and scattered positive cells in the alveoli were noted (Figure 6C).

Spleen, Tonsils, Lymph Nodes, and Bone Marrow

Some positive cells, possibly dendritic, in the germinal centers of the follicles, mainly in the spleen, were found. Various inflammatory cells, mainly neutrophils but also some plasma cells, also showed positivity.

Thymus

Strong positivity in the Hassall's corpuscles was characteristic (Figure 6D).

Adrenal Gland

Weak to moderate positivity was observed in the medulla.

Thyroid Gland

Focal protein immunoexpression was revealed by follicular cells, mainly in hyperplastic conditions and in oxyphilic cell metaplasia (Figure 7A).

Parathyroids

Immunoexpression by the oxyphilic cells was noted (Figure 7B).

Pituitary Gland

In the anterior pituitary, many cells expressed the protein strongly (Figure 7C). The pituitocytes of the pars nervosa were negative.

Mesothelium (Pleura)

The positivity was variable and was observed mainly in hyperplastic conditions.

Squamous Epithelia

Squamous epithelia were generally negative. In some cases weak focal expression by keratinocytes (cervix, mouth mucosa) was observed but could not be considered safely as positivity.

Mesenchymal Tissues

In general, mesenchymal tissues, except for neural tissue, were negative. Some weak positivity was observed in some myoepithelial cells in the walls of small arteries and in chondrocytes (Figure 7D).

Diffuse Neuroendocrine System

Neuroendocrine cells in several organs expressed hK6.

In short, hK6 is expressed by many normal human tissues. Glandular epithelia constitute the main hK6 immunoexpression sites, with representative organs being breast, prostate, kidney, endometrium, colon, appendix, salivary ducts, bile ducts, and gallbladder. The small intestine, the stomach, the endocervix, the Fallopian tube, the epididymis, the bronchus, and the upper respiratory tract showed focal expression. Choroid plexus epithelium, peripheral nerves, and neuroendocrine cells (including the islets of Langerhans and adrenal medulla) expressed the protein strongly and diffusely. Characteristic immunostaining was observed in Hassall's corpuscles of the thymus, oxyphilic cells in the thyroid and parathyroid glands, in the primordial follicles of the ovary, in dendritic cells mainly in the spleen, and in various cells of the placenta.

Discussion

The kallikrein 6 gene (KLK6) is one of the recently cloned members of the human tissue kallikrein gene family (Diamandis et al. 2000a). Messenger RNA encoding for hK6 protein (zyme/protease M/neurosin) has been previously detected in many tissues, including breast, brain, spinal cord, cerebellum, kidney, uterus, salivary gland, thymus, spleen, and testis in some mammalian species but not in mice, rats, or hamsters (Anisowicz et al. 1996; Little et al. 1997; Yamashiro et al. 1997; Yousef et al. 1999). Until recently, no methods were available for the quantitative measurement of hK6 in biological fluids and tissues.

Figure 3 (A) Droplet-like cytoplasmic and brush-border immunostaining for hK6 in the endometrium (polyclonal antibody). (B) Strong diffuse immunoexpression of hK6 by the prostate columnar epithelium (polyclonal antibody). (C) Strong diffuse immunoexpression of hK6 by the prostate columnar epithelium (monoclonal antibody). (D) Unstained prostate epithelium with non-immune rabbit serum. Original magnifications \times 200.

Figure 4 (A) hK6 immunoexpression by the urinary tubuli of the kidney (polyclonal antibody). (B) Subnuclear cytoplasmic hK6 immunostaining in the columnar epithelium of the colon (polyclonal antibody). (C) Subnuclear, brush-border, and parietal cell hK6 immunoexpression in the gastric mucosa (polyclonal antibody). (D) hK6 immunoexpression in a focus of intestinal metaplasia in the gastric mucosa (monoclonal antibody). Original magnifications ×200.



The development of polyclonal antibodies and of an immunofluorometric procedure for quantifying the hK6 protein has helped to define the organs that express this protein and its relationship to cancer and other pathological conditions (Diamandis et al. 2000c, d,e). We used recombinant hK6 protein for the development of polyclonal rabbit and mice antibodies and monoclonal mouse antibodies. The developed immunoassay for hK6 (Diamandis et al. 2000c) demonstrates good sensitivity and dynamic range and detects a single immunoreactive band in all biological fluids examined. This proteinase is present in its free form in serum, in contrast to PSA, which is mainly bound to α_1 -antichymotrypsin (Stenman et al. 1991; Diamandis et al. 2000c).

In our recent work, we found the highest concentration of hK6 in milk of lactating women, followed by cerebrospinal fluid, nipple aspirate fluid, and breast cyst fluid (Diamandis et al. 2000c). The protein was also detected in male and female serum, in the majority of seminal plasmas, and in a relatively small percentage of amniotic fluids and breast tumor cytosolic extracts, but not in urine. The examination of a number of human tissue extracts revealed high concentrations of hK6 in the salivary glands, lung, colon, Fallopian tube, placenta, breast, pituitary, and kidney. We further found that hK6 concentration in brain tissue extracts from patients with Alzheimer's disease was lower than that in normal controls; however, the CSF and serum hK6 concentrations were higher in patients with the disease (Diamandis et al. 2000e). Using the rabbit polyclonal antibody, we localized hK6 by IHC in normal epithelial cells of the choroid plexus. Therefore, hK6 may constitute a new biomarker for diagnosis and monitoring of Alzheimer's disease (Diamandis et al. 2000e).

This is the first report describing IHC localization of hK6 in a large variety of human tissues. Only Little et al. (1997) have previously studied hK6 immunohistochemically, and only in brain tissues. Because we used exclusively normal tissues, it will be interesting to follow up these studies with tissues from various pathological conditions, to establish if hK6 has value as a disease biomarker.

hK6 was localized in a large number of normal human tissues and therefore cannot be considered as a

specific tissue marker, in contrast to the homologous proteins hK2 and PSA, which show prostate-restricted specificity (McCormack et al. 1995; Rittenhouse et al. 1998). With the availability of highly sensitive techniques, it has become apparent that these two kallikreins are also expressed in many other tissues, including breast, thyroid, and salivary glands (Black and Diamandis 2000; Maglara et al. 2000). The expression of hK6 mainly by glandular epithelia suggests that it is secreted. This is further supported by the finding of hK6 in many biological fluids (Diamandis et al. 2000c). The absence of hK6 in urine corresponds well with the lack of immunoreactivity by the urothelium. The positive immunoexpression of glial cells in our study is in accordance with previous findings (Little et al. 1997; Yamanaka et al. 1999). Yamanaka et al. (1999) found KLK6 mRNA in mature oligodendrocytes and suggested that this protease may be important in the processes occurring after maturation of oligodendrocytes, such as myelination or turnover of the proteins in the myelin. Little et al. (1997) detected positive immunostaining 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. We localized hK6 also in the choroid plexus epithelium and correlated our findings with Alzheimer's disease (Diamandis et al. 2000e). Yamashiro et al. (1997) found strongest KLK6 mRNA expression in brain, followed by expression in spleen, in accordance with the immunoreactivity of the Hassall's corpuscles of the spleen in our study. An interesting finding is the expression of hK6 in oxyphilic cells in the thyroid and parathyroid glands. This accords with demonstration of hK2 and hK3 in oxyphilic cells of the thyroid (Maglara et al. 2000), suggesting another similarity among these three kallikreins. The detection of hK6 in human male and female serum (Diamandis et al. 2000c) is indicative that it is secreted by various tissues. Worth mentioning is the previous demonstration of other kallikreins, including hK3 (PSA) and hK2, in breast secretions, breast cancer cytosols, seminal plasma, and amniotic fluids (Yu and Diamandis 1995a,b; Black et al. 2000; Black and Diamandis 2000).

Another interesting finding was the presence of hK6 in some cells of the diffuse neuroendocrine sys-

Figure 5 (A) Strong immunohistochemical expression of hK6 in the islets of Langerhans in the pancreas (polyclonal antibody). (B) Strong immunohistochemical expression of hK6 in the islets of Langerhans in the pancreas (monoclonal antibody). (C) Unstained islets cells after antibody immunoabsorption. (D) Unstained islets cells with non-immune rabbit serum. Original magnifications ×200.

Figure 6 (A) Cytoplasmic and brush-border hK6 immunostaining in the epithelium of the gallbladder (polyclonal antibody). (B) Cytoplasmic hK6 immunoexpression by the bronchial epithelium (polyclonal antibody). (C) Cytoplasmic immunohistochemical expression of hK6 mainly in the duct epithelium of the major salivary ducts (polyclonal antibody). (D) Strong immunohistochemical staining for hK6 in the Hassall's bodies of the thymus (polyclonal antibody). Original magnifications \times 200.



Figure 7 (A) hK6 cytoplasmic immunoexpression by follicular cells in the thyroid gland (polyclonal antibody). (B) hK6 immunoexpression by oxyphilic cells in the parathyroid glands (polyclonal antibody). (C) Cytoplasmic hK6 immunostaining by cells of the anterior pituitary (monoclonal antibody). (D) Immunostaining of hK6 by chondrocytes (polyclonal antibody). Original magnifications ×200.

tem and in nerves. The staining was relatively strong, and we believe that it could be representative of neuroendocrine differentiation. The expression of hK6 is reminiscent of CD56 (natural killer cell-associated antigen, neural cell adhesion molecule) and CD57 (Leu-7, T-cell surface marker) which are sensitive but not specific for cells and neoplasms with neuroendocrine differentiation (Kaufmann et al. 1997). These suggestions need verification with a study of a variety of neuroendocrine tumors. Furthermore, we found strong hK6 expression in the islets of Langerhans and in the epithelium of the pancreatic ducts, but not in the acinar cells of the exocrine pancreas. It is possible that hK6 is involved in islet hormone processing, a property already attributed to many other proteolytic enzymes (Seidah and Chretien 1999).

The contribution of hK3 (PSA) and hK2 in the diagnosis and monitoring of prostate cancer suggests that other kallikreins may also have value as candidate biomarkers. We have already shown that serum hK6 concentration is increased in ovarian carcinoma (Diamandis et al. 2000d). Some other kallikrein genes are either underexpressed or overexpressed in certain car-

cinomas (Yousef and Diamandis 2001). The encoded proteases may serve to positively or negatively regulate cell growth or differentiation by cleavage of cell surface receptors (Coughlin 1999) or cell growth regulatory and angiogenic proteins, and by activation of other proteases for invasion and metastasis. These serine proteases are secreted into the extracellular space and therefore are excellent candidate circulating tumor markers. In addition to KLK2 and KLK3, other kallikrein genes have been studied in relation to oncogenesis and prognosis of malignant tumors (Diamandis et al. 2000a; Yousef and Diamandis 2001). Liu et al. (1996) reported downregulation of KLK10 in cancerous breast and prostate tissues and in cancer cell lines. Others reported upregulation of TADG-14/ KLK8 and KLK7 in ovarian carcinomas (Tanimoto et al. 1999; Underwood et al. 1999). Breast, prostate, renal, and gastrointestinal cancer may reveal some relation to hK6 expression, as these organs strongly express this protein.

KLK6 has been shown to be regulated by steroid hormones (Yousef et al. 1999). It will be interesting to study steroid hormone-responsive organs for hK6 expression and to investigate its possible involvement in tumor development, progression, or both (Diamandis et al. 2000a; Yousef and Diamandis 2001). Some kallikreins might be good therapeutic targets and the enzymatic activity of these proteins might be beneficial or deleterious. The development of tissue kallikrein inhibitors or activators may provide a new generation of drugs against cancer and other disorders.

Literature Cited

- Anisowicz A, Sotiropoulou G, Stenman G, Mok SC, Sager R (1996) A novel protease homolog differentially expressed in breast and ovarian cancer. Mol Med 2:624–636
- Black MH, Diamandis EP (2000) The diagnostic and prognostic utility of prostate specific antigen for diseases of the breast. Breast Cancer Res Treat 59:1–4
- Black MH, Magklara A, Obiezu C, Levesque MA, Sutherland DJ, Tindall DJ, Young CY, Sauter ER, Diamandis EP (2000) Expression of a prostate-associated protein, human glandular kallikrein (hK2), in breast tumors and in normal breast secretions. Br J Cancer 82:361–367
- Chu TM (1997) Prostate-specific antigen and early detection of prostate cancer. Tumor Biol 18:123–134
- Coughlin SR (1999) How the protease thrombin talks to cells. Proc Natl Acad Sci USA 96:11023–11027
- Diamandis EP (1999) Prostate specific antigen—its usefulness in clinical medicine. Trends Endocrinol Metab 25:14–16
- Diamandis EP, Yousef GM, Clements J, Ashworth LK, Yoshida S, Egelrud T, Nelson PS, Shiosaka S, Little S, Lilja H, Stenman U-H, Rittenhouse HG, Wain H (2000b) New nomenclature for the human tissue kallikrein gene family. Clin Chem 46:1855–1858
- Diamandis EP, Yousef GM, Luo LY, Magklara A, Obiezu CV (2000a) The new human kallikrein gene family: implications in carcinogenesis. Trends Endocrinol Metab 11:54–60
- Diamandis EP, Yousef GM, Petraki C, Soosaipillai AR (2000e) Human kallikrein 6 as a biomarker of Alzheimer's disease. Clin Biochem 33:663–667
- Diamandis EP, Yousef GM, Soosaipillai AR, Bunting P (2000d) Human kallikrein 6 (zyme/protease M/neurosin): a new serum biomarker of ovarian carcinoma. Clin Biochem 33:579–583
- Diamandis EP, Yousef GM, Soosaipillai AR, Grass L, Porter A, Little S, Sotiropoulou G (2000c) Immunofluorometric assay of human kallikrein 6 (zyme/protease M/neurosin) and preliminary clinical applications. Clin Biochem 33:369–375
- Kaufmann O, Georgi T, Dietel M (1997) Utility of 123C3 monoclonal antibody against CD56 (NCAM) for the diagnosis of small cell carcinomas on paraffin sections. Hum Pathol 28:1373–1378
- Little SP, Dixon EP, Norris F, Buckley W, Becker GW, Johnson M, Dobbins JR, Wyrick T, Miller JR, MacKellar W, Hepburn D, Corvalan J, McClure D, Liu X, Stephenson D, Clemens J, Johnstone EM (1997) Zyme, a novel and potentially amy-

- Liu XL, Wazer DE, Watanabe K, Band V (1996) Identification of a novel serine protease-like gene, the expression of which is downregulated during breast cancer progression. Cancer Res 56:3371– 3379
- Maglara A, Cheung CC, Asa SL, Diamandis EP (2000) Expression of prostate-specific antigen and human glandular kallikrein 2 in the thyroid gland. Clin Chim Acta 300:171–180
- McCormack RT, Rittenhouse HG, Finlay JA, Sokoloff RL, Wang TJ, Wolfert RL, Lilja H, Oesterling JE (1995) Molecular forms of prostate-specific antigen and the human kallikrein gene family: a new era. Urology 45:729–744
- Rittenhouse HG, Finlay JA, Mikolajczyk SD, Partin AW (1998) Human kallikrein 2 (hK2) and prostate-specific antigen (PSA): two closely related, but distinct, kallikreins in the prostate. Crit Rev Clin Lab Sci 35:275–368
- Seidah NG, Chretien M (1999) Properties and phohormone convertases: a family of subtilases generating diverse bioactive polypeptides. Brain Res 848:45–62
- Stenman U-H (1999) New ultrasensitive assays facilitate studies on the role of human glandular kallikrein (hK2) as a marker for prostatic disease. Clin Chem 45:753–754
- Stenman U-H, Leinonen J, Alfthan H, Rannikko S, Tuhkanen K, Alfthan O (1991) A complex between prostate-specific antigen and alpha1-antichymotrypsin is the major form of prostate-specific antigen in serum of patients with prostate cancer: assay of the complex improves clinical sensitivity for cancer. Cancer Res 51:222–226
- Tanimoto H, Underwood LJ, Shigemasa K, Yan Yan MS, Clarke J, Parmley TH, O'Brien TJ (1999) The stratum corneum chymotryptic enzyme that mediates shedding and desquamation of skin cells is highly overexpressed in ovarian tumor cells. Cancer 86:2074–2782
- Underwood LJ, Tanimoto H, Wang Y, Shigemasa K, Parmley K, O'Brien TJ (1999) Cloning of tumor-associated differentially expressed gene-14, a novel serine protease overexpressed by ovarian carcinoma. Cancer Res 59:4435–4439
- Yamanaka H, He X, Matsumoto K, Shiosaka S, Yoshida S (1999) Protease M/neurosin mRNA is expressed in mature oligodendrocytes. Brain Res Mol Brain Res 71:217–224
- Yamashiro K, Tsuruoiko N, Kodama S, Tsujimoto M, Yamamura T, Tanaka T, Nakazato H, Yamaguchi N (1997) Molecular cloning of a novel trypsin-like serine protease (neurosin) preferentially expressed in brain. Biochim Biophys Acta 1350:11–14
- Yousef GM, Diamandis EP (2001) The new human tissue kallikrein gene family: structure, function and association to disease. Endocrine Rev 22:184–204
- Yousef GM, Luo L-Y, Scherer SW, Sotiropoulou G, Diamandis EP (1999) Molecular characterization of zyme/proteaseM/neurosin (PRSS9), a hormonally regulated kallikrein-like serine protease. Genomics 62:251–259
- Yu H, Diamandis EP (1995a) Prostate specific antigen in milk of lactating women. Clin Chem 41:54-58
- Yu H, Diamandis EP (1995b) Prostate specific antigen immunoreactivity in amniotic fluid. Clin Chem 41:204–205