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Human Tissue Kallikreins – A Family with many surprises

[İnsan Doku Kallikreinleri – Sürprizlerle Dolu Bir Enzim Ailesi]

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ABSTRACT

The human tissue kallikreins (KLKs) form a family of 15 closely related serine proteases. They are encoded by conserved genes tandemly located in a large gene cluster (320 kb) on chromosome 19q13.4. The first three members of the family, KLK1 (tissue kallikrein), KLK2, and KLK3 (Prostate specific antigen, PSA) were long thought to be the only members of the family. However, during the last decade the availability of human genome sequence and extensive screening of the KLK locus has revealed the presence of 12 additional KLK genes. With the complete description of the human KLK locus, the main research effort is now centered around the elucidation of potential biological functions of the KLKs. PSA/KLK3 is a well known biomarker for prostate cancer; KLK2 is also being considered as a marker for this disease. At present, other members of the KLK family are under investigation as potential markers in disease states. In fact, several have shown potential as prognostic biomarkers, especially in hormone dependent cancers, such as those involving the prostate, breast and ovary. Although the biological function of KLKs are still in large part unknown, they are expressed in a wide range of tissues, suggesting a functional role in diverse physiological and pathophysiological processes. Among others, these include skin desquamation and other skin diseases, tooth development and enamel defects, Alzheimer's disease, and Parkinson's disease. An increasing interest in the role of KLKs in disease has also resulted in research into potential substrates of the KLKs, which may give clues to their functional role. In vitro studies have shown that some KLKs can auto-activate, while others can activate each other, suggesting that the KLKs may be part of an enzymatic cascade. Further research will reveal the functional roles of KLKs in various tissues, and whether they have clinical utility as biomarkers for disease states, and possibly also as therapeutic targets.

Key Words: kallikreins, tissue endopeptidase, gene family, cancer, tumor markers

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ÖZET

İnsan dokusu kallikreinleri 15 adet yakın ilişkili serin proteazdan oluşan bir aileyi meydana getirir. 19q13.4 kromozomu üzerinde birbiri ardına yerleşmiş korumalı geniş bir gen kümesi (320 kb) tarafından şifrelenir. Ailenin ilk üç üyesi olan, KLK1 (doku kallikreini), KLK2, ve KLK3 (Prostat spesifik antijen, PSA) uzun zaman boyunca aileyi oluşturan başlıca üyeler olarak düşünüldü. Oysa, son on yılda insan genom dizisinin çözülmesi ve KLK bölgesinin kapsamlı görüntülenmesi, ekstra 12 KLK geninin varlığını ortaya çıkarmıştır. İnsanda KLK bölgesinin bütünüyle tamamlanması ile araştırmaların ana gayesi KLK'lerin potansiyel biyolojik fonksiyonlarını açığa çıkarmak olarak belirlenmiştir. PSA/KLK3 prostat kanseri teşhisinde iyi bilinen bir biyolojik işaretleyicidir. KLK2 de benzer şekilde aynı hastalığın belirteci sayılmaktadır. Şu an, KLK ailesinin diğer üyeleri hastalık tespitinde potansiyel belirleyici olma özellikleri açısından incelenmektedirler. Gerçekte, pek çoğu, özellikle hormonlara bağlı meme, prostat ve yumurtalık kanseri türlerinde tahmini belirteçler olarak potansiyel taşımaktadırlar. Her ne kadar KLK'lerin biyolojik fonksiyonu hala büyük oranda bilinmiyor olsa da, doku sınıfı altında geniş bir perspektifte ifade edilmekte ve çeşitli psikolojik ve pato-fizyolojik. süreçlerde fonksiyonel rol taşımaktadırlar. Alzheimer ve Parkinson hastalıkları, diş gelişimi problemleri, diş minesinde bozulmalar, dokularda teşekkül eden hastalıklar ve derinin pul pul dökülmesi gibi rahatsızlıklarda rol oynadıkları düşünülmektedir. KLK'lerin hastalıkların meydana gelmesindeki etkileri üzerinde yapılan araştırmalar gün geçtikçe artmaktadır. KLK'lerin biyokimleri üzerindeki çalışmalar da fonksiyonel rollerinin belirlenmesi yolunda önem taşımaktadır. In-vitro çalışmalar göstermektedir ki, bazı KLK'ler kendiliğinden aktive olabilirken diğerleri birbirlerini aktif hale getirebilmektedirler. In-vitro çalışmalar ortaya koymuştur ki, bazı KLK'ler enzimlerden oluşan kademeli dizilerin bir parçası olabilir. Bu konuda ilerlemekte olan çalışmalar ışığında farklı dokularda KLK'lerin fonksiyonel rolleri açığa çıkacak ve hastalık tanısının oluşmasında biyolojik belirteç olarak ve tedavi hedefleriyle ilgili klinik faydalanımları bulunup bulunmadığı ortaya çıkacaktır.

Anahtar kelimeler: Kallikreinler, doku endopeptidazı, gen ailesi, kanser, tümör belirteçleri

INTRODUCTION

History

The first member of the tissue kallikrein family was identified in the 1930's as an abundant protein in pancreas, hence the name kallikrein, derived from the Greek word for pancreas "kallikreas" (1,2). Initially, two distinct types of kallikreins were noted - a circulating, liver-derived plasma kallikrein, and a glandular or tissue kallikrein (3,4). The plasma kallikrein, or *KLKB1* gene, has no related counterparts. Although the tissue kallikrein (KLK1) protein was discovered in the 1930's, its gene (*KLK1*) was not discovered until 1985 (5,6). The next two genes of the family, *KLK2* and *KLK3/PSA*, were discovered during the late 1980s (7-9), and at this time it was concluded that the human kallikrein family had only three members. Not until 1994 did we see the expansion of the human kallikrein family, which expanded to 15 members during the next 7 years as the complete human kallikrein locus was sequenced and characterized.

The human kallikrein locus

After the cloning of the classical kallikrein genes (*KLK1-KLK3*), the remaining members of the family were identified based on their sequence similarity to these. The 15 members of the family are predicted to encode homologous serine proteases from a locus of 15 structurally similar genes (*KLK1-KLK15*) that tandemly localize to chromosome 19q13.4 in humans (10-12). This gene cluster represents the largest cluster of contiguous protease genes in the entire genome (13,14). The human kallikrein locus has its rodent counterpart with a cluster of 25 functional genes in mouse (15,16) and 10 functional genes in rat (17). The significance of the different numbers of *KLK* genes in the different organisms is currently not known. Tissue kallikrein proteins have also been identified in a variety of additional species (for review see e.g. (18)). In general, mammalian *KLK* loci contain a single copy of *KLK4-KLK15* genes, and varying numbers of the classical *KLK* genes and pseudogenes.

Figure 1 shows a schematic presentation of the human kallikrein locus. For details about the human kallikrein

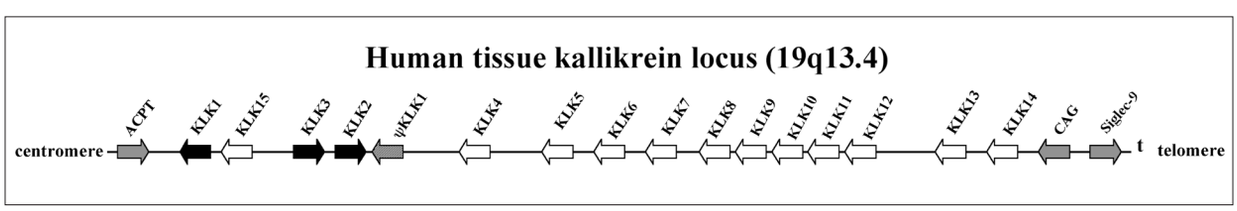

Figure 1. Human tissue kallikrein gene locus at chromosome 19q13.4. Arrowheads indicate the location of genes and their direction of transcription. Black arrowheads: the classical kallikreins KLK1-KLK3; white arrowheads: the remaining 12 KLK encoding genes: striped arrowhead: the KLK1 pseuodogene; gray arrowheads: non-kallikrein genes ACPT, CAG and Siglec9. The official gene names are indicated above each arrowhead. The orientation is shown centromere to telomere. Modified from (79) and (153).

gene cluster, see recent reviews (11,19). The KLKs have significant sequence similarities at the DNA and amino acid levels (40% to 80%) (20). The structural organization and size of the new kallikrein genes (*KLK4-KLK15*) is similar to that of other *KLK* genes except for additional exons encoding 5' or 3' untranslated regions. Moreover, many of these genes have multiple mRNA transcripts, a trait not observed with rodent genes. Alternative splicing, as a result of splicing in the coding or non-coding regions, use of different transcription or translation start and stop sites, and also combinations of these, is actually prevalent within the human kallikrein locus. Alternative splice variants have been described for all but one (*KLK14*) of the *KLK* genes. These variants also possibly result in different protein products, which may have important functional roles in the cells in which they are expressed and may be important in the search for biomarkers and possible drug targets. For a review about the alternative transcripts of the *KLK* genes, see (21).

Biochemical properties of human kallikreins

The proteins encoded by the KLK gene locus share several structural characteristics. They are all potential serine proteases, and the location of the start, stop and catalytic histidine, aspartate, and serine codons are remarkably similar (19). The classical KLK proteins, KLK1-KLK3, have a sequence homology of 62-77%, while the more recently identified KLK4-KLK15 proteins are less related at the amino acid level (25-66%) (22). The key residues (His, Asp and Ser) that denote serine protease activity are, however, entirely conserved. The human KLKs are translated as pro-enzymes, containing a signal peptide of 16 to 30 amino acids at their NH_2 terminus, targeting them for secretion. The signal peptide is followed by a pro-peptide of 4-9 amino acids. At least one of the KLKs, namely KLK4, is also expressed without this signal peptide, giving rise to an intracellular, predominantly nuclear protein (23). The calculated molecular weight of the peptide moiety of pro-KLK proteins ranges from ~23 to ~26 kDa. Due to glycosylation and potentially other post-translational modifications, larger masses have been observed for several kallikreins. In KLK1, O-linked glycosylation has been observed (24), while all other reports involve the addition of N-linked carbohydrates. Prediction of glycolysation sites in the human proteome (25) indicate that most KLKs contain one or more putative N-glycosylation sites (Asn-X-Ser/Thr) while only a few have potential Ser/Thr residues involved in O-linked glycosylation.

Mechanism of action

The KLK proteins were first described for their kininogenase activity that generates the vasoactive peptides bradykinin (BK) or kallidin (Lys-BK) from kininogens. Today, the tissue kallikreins have come to denote a group of structurally related serine proteases that are part of the specific gene cluster, mainly due to sequence and structural similarity, and they are not necessarily enzymes with kininogenase activity. In fact, it appears that in the KLK family, it is only KLK1 which has significant kininogenase activity (26).

Proteases are enzymes that catalyze peptide bond hydrolysis, and they perform fundamental functions in all living organisms. The complete set of proteases (or degradome) expressed at a given time within a cell, tissue, or organism comprises ~2% of all genes in many organisms (27). In general, the members of the KLK family are serine proteases with trypsin and/or chymotrypsin activity. The highly conserved active site triad of the three amino acids His, Asp and Ser, act as a charge relay system for proteolytic catalysis. The amino acid of the substrate-binding pocket (S1), located six residues NH_2-terminal of the catalytic serine (Ser189 according to chymotrypsin numbering), determines their substrate cleavage specificity. In general this residue is either an Asp183 (trypsin-like) or Ser183 (chymotrypsin-like), with preference for basic or hydrophobic amino acids, respectively. The majority of the KLKs appear to be trypsin-like in action due to an asparagine or glutamate in this position, while KLK3, KLK7 and KLK9 have non-polar residues in this position denoting a chymotrypsin-like action (28,29). Ten of the disulphide bond-forming cysteines are absolutely conserved among the 15 kallikreins.

X-ray crystal structures have been resolved for mature KLK1 (30) and mature and pro-KLK6 (31,32), in addition to several non-human kallikreins (33-36). Figure 2 shows the crystal structure of mature KLK6 (32), with the active site cleft made up of the catalytic triad (His57, asp102, and Ser195) and the S1 site (Asp192). In general, KLKs possess archetypal tertiary

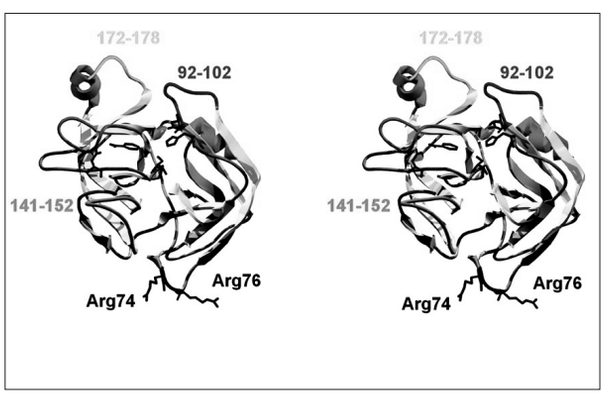

Figure 2. Stereo ribbon diagram of human KLK6. The orientation is intended to show the active site cleft with the catalytic triad (His57, Asp102, and Ser195), the S1 site (Asp192) and bound benzamidine inhibitor. The location of loop regions is shown; 92-102 (blue), 141-152 (magenta), and 172-178 (green) that border the active site. Modified from original figure (32) with permission.

structure of trypsin/chymotrypsin serine proteases, with two juxtaposed six-stranded antiparallell β-barrels and two α-helices, with the active site bridging the barrels (37,38). A variable external surface loop surrounds the S1 site. KLK1-3 possesses a unique surface loop called the "kallikrein loop", which is not present in its entirety in any of the other KLKs and absent in other serine proteases. Regulation of KLK activity may be achieved by glycosylation of the kallikrein loop (39,40).

Biological function and role in disease

The KLKs are expressed in a wide array of tissues, mainly those that are under steroid hormone control (10,11). To date, there is substantial, although not final, information on the biological function of KLK1-3, the first family members to be cloned. The functions of the remaining KLKs, KLK4 to KLK15, are under investigation and are at present poorly understood.

KLK1

Of all the KLKs, KLK1 has the most efficient kininogenase activity. KLK1 cleaves two specific peptide bonds of plasma kininogen, Met/Lys and Arg-Ser (26), to yield the vasoactive peptide hormone Lys-bradykinin (kinin or kallidin). KLK1 thus plays an important role in the kallikrein-kinin system (KKS), which is the key proteolytic system participating in control of a wide spectrum of physiological functions, including blood pressure, regulation of smooth muscle contractility of some organs, increase of vascular permeability and the development of inflammation (41-43). KLK1 is therefore associated with a broad range of human diseases affected by the KKS, such as hypertension (44), inflammation (45) and malignancy (46). In addition, KLK1 can process some biologically active peptides, including growth factors and pro-insulin (47). Relevant to cancer, the kinin receptor has been found in astrocytic tumor cells (48) and the bradykinin receptor has been found in endometrial and prostatic carcinomas (46), while the KLK1 transcripts were detected in renal cell carcinoma (49).

KLK2

Unlike KLK1, KLK2 has low kininogenase activity (50,51). KLK2 has been shown to activate the urokinase type plasminogen activator (uPA) (52), and inactivates plasminogen activator inhibitor-1 (PAI-1) (53). In addition, KLK2 can activate the conversion of pro-PSA to mature, enzymatically active PSA, thus implying that KLK2 may be regulating PSA activity *in vivo*, and that these two kallikreins may act in concert in extraprostatic locations (54). However, this fact is still debated (55). In a recent study by Hekim and coworkers, enzymatically active KLK2 was used to screen phage display peptide libraries for KLK2 inhibitors. Three peptides were found to be specific and efficient inhibitors of the enzymatic activity of KLK2 towards a peptide substrate, and also inhibited the activation of the pro-form of PSA (56). Being a trypsin-like serine protease, KLK2 can hydrolyze the pro-KLK2 substrate suggesting its autoprocessing capacity (57). Prostate is the most KLK2 enriched organ, which implies the potential biological role of KLK2 in male fertility. Seminal KLK2 can cleave semenogelin I and II in seminal fluid (58). Other studies have suggested that KLK2 may also be involved in processing of biological peptides (59). KLK2 has recently become one of the most promising biomarkers for prostate cancer in addition to PSA (60). Combined with PSA, KLK2 may improve the detection of prostate cancer (61) and aid in the discrimination between prostate cancer and benign prostatic hyperplasia (62). Recent studies demonstrate that KLK2 could also help in determining the stage of prostate cancer (63), as well as predict prognosis (64). Although KLK2 was also detected in breast cancer and other malignancies, there is limited information on the potential clinical value of KLK2 in cancers other than prostate cancer (65).

KLK3/PSA

The major substrates of PSA are semenogelin I, II and fibronectin (58,66). PSA hydrolyses the seminal vesicle proteins semenogelin I and II in ejaculate, thereby contributing to the liquidification of the seminal fluid, an event that is integral to sperm motility (58,67). PSA has been implicated in growth regulation by the cleavage of IGFBP3 (68) and PTHrp (69,70), proteins associated with cellular growth regulation and breast cancer metastasis, respectively. PSA is synthesized as a zymogen (pro-PSA) (71) and can be activated by KLK2 (54,72,73) or more efficiently by prostin (74). KLK4 may also be an activator of pro-PSA; however, this was only shown for a chimeric form of KLK4 (75). In addition, a recent study also suggests KLK5 to be a possible activator of pro-PSA (76). Although PSA exhibits a chymotrypsin activity, it can not activate itself by autoprocessing (77). PSA is produced mainly by the prostate ductal and acinar epithelium and secreted into the lumen, and is a well known serum marker for the detection and progression of prostate cancer (for review, see e.g. (78,79)). It has been widely used in prostate cancer screening (80), detection (81), diagnosis, treatment selection and monitoring (82). However, due to high rates of false positives and false negatives, its use for screening purposes is highly debated (for review see e.g (83)). Recent studies have shown that PSA is expressed in both male and female breast cancer (84,85); in contrast to prostate cancer, it may be a marker for favorable prognosis in breast cancer (86).

KLK4

The first member of the extended kallikrein family, KLK4, was cloned in 1999 and is also known as prostase, KLK4-L1, PRSS17 and ARM-1 (87-90). The *KLK4* gene

gives rise to at least 8 different mRNA forms through alternative splicing and/or alternative transcription start site (90). These transcripts are expected to give rise to at least 7 different protein moieties (90) (21), however, only two encoded proteins have been identified to date; one intracellular, predominantly nuclear form (23,91) and one cytoplasmic, secreted form (91-93). The relative abundance of the two isoforms is presently unclear, although in quantitative RT-PCR experiments the truncated form appears to be at least 100-fold more abundant than the longer, secreted form (Xi et al., 2004). The truncated, predominantly nuclear form of KLK4 suggest that it has a different function compared with other KLKs (23,91). Takayama and coworkers showed that a recombinant, chimeric form of KLK4 (ch-KLK4) in which the pro-piece of KLK4 was replaced by that of PSA to create an activation site susceptible to trypsin-type proteases, was able to cleave the chromogenic substrates Val-Leu-Arg-pNA (S-2266), Pro-Phe-Arg-pNA (S-2302), Ile-Glu-Gly-Arg-pNA (S-2222), and Val-Leu-Lys-pNA (S-2251), indicating that ch-KLK4 has a trypsin-type substrate specificity (75). In addition, ch-KLK4 also readily activated both pro-PSA and single chain urokinase-type plasminogen activator (scuPA, pro-uPA), and completely degraded prostatic acid phosphatase, indicating that KLK4 may have a role in the physiologic processing of seminal plasma proteins, as well as in the pathogenesis of prostate cancer through its activation of pro-uPA (75). In a recent report, it was demonstrated that the three-domain receptor of uPA, uPAR, is also a target for KLK4, cleaved in the D1-D2 linker sequence and, to a lesser extent, in its D3 juxtamembrane domain (94). These data suggest a role of KLK4 in modulation of the tumor-associated uPA/uPAR-system activity by either activating pro-uPA or cleaving the cell surface-associated uPA receptor. In mice, KLK4 has been found to regulate enamel matrix protein processing and further function in defining structure and composition of enamel (95). Recent studies reported that mutation of KLK4 result in enamel defect (96,97). KLK4 is highly prostate enriched, and is androgen regulated (23,88-90), which suggests that it may function in seminal fluid liquefaction similar to KLK2 and PSA (11,87). However, until today, there is not sufficient substrate and enzymatic evidence to support the notion that KLK4 has a functional role in seminal liquefaction, although a secreted form of KLK4 has been detected in seminal plasma (91,92).

In situ hybridization analysis on tissue microarrays showed an increased *KLK4* mRNA expression in prostate cancer as compared to the normal prostate; however, there was no association with tumor stage or grade (23), Figure 3. This is consistent with KLK4 protein staining results (our unpublished data). Veveris-Lowe showed that KLK4, as well as PSA, increases cell migration when expressed in the prostate cancer cell line PC3 and this was associated with loss of E-cadherin and increase of vimentin (93). These results suggest that KLK4 may have a role in prostate cancer development and progression.

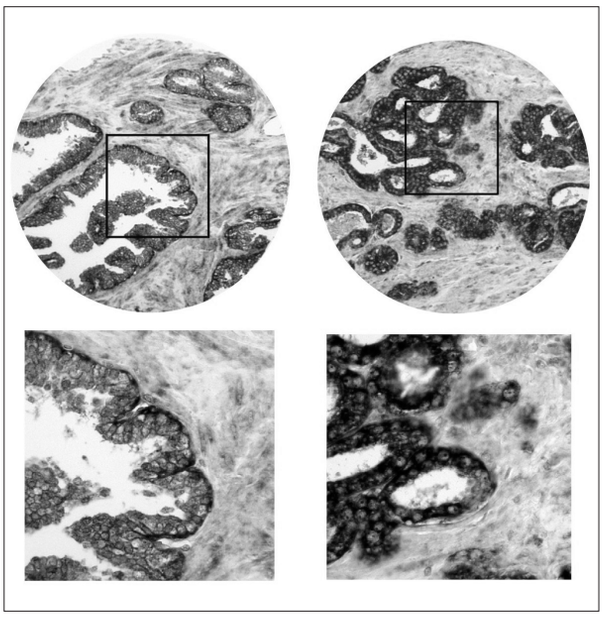

Figure 3. KLK4 mRNA expression in normal and malignant prostate. A KLK4-specific ribonucleotide probe was used for in situ hybridization on a tissue microarray with both normal and tumor glands from human prostates. The KLK4 mRNA is predominantly detected in the basal cells of the normal prostate epithelium (left), with more unordered and intensive expression in the tumors (right). Adapted from (23) with permission.

Although KLK4 expression is highly enriched in the normal prostate compared with other tissues, it is also highly expressed in ovarian tumors (55,98,99). KLK4 has been suggested to be a poor prognosis factor for serous ovarian cancer (98,100). Furthermore, a recent study has shown that KLK4 is overexpressed in ovarian cancer effusions, and this may be related to loss of stromal contribution and/or altered microenvironment (101). In addition, KLK4 has been implicated in paclitaxel resistance of ovarian cancer (99).

KLK5

KLK5 is a trypsin-like protease, and recombinant KLK5 is inhibited by LEKT1 (lympho-epithelial Kazal-type inhibitor), a serine protease inhibitor (102). KLK5, which has been cloned from stratum corneum and is abundant in skin tissue (103), is proposed to have a function in the degradation of intercellular structure (104), thereby monitoring the skin cell shedding or desquamation during epidermal turnover (105,106). In a recent study with recombinant kallikreins, it was shown that KLK5 can activate pro-KLK2 and pro-PSA, and thereafter internally cleave and deactivate active KLK2 and PSA. This suggests that KLK5 may be a member of a proteolytic cascade pathway involved in seminal clot liquefaction (76). Even though KLK5 function is restricted mainly to skin and is involved in skin desquamation and skin diseases (104), studies so far have

focused more on its potential as a possible biomarker for cancer than on the relation between KLK5 and dermal disease. KLK5 overexpression has been shown to be related to the poor prognosis of breast and ovarian cancer (107-109). Another report found higher KLK5 expression in lung squamous cell carcinoma compared with in matched nonmalignant lung tissue (110).

KLK6

The mature recombinant KLK6 was found to have a trypsin-like activity against synthetic substrates (111). Human plasminogen was identified as a putative physiological substrate for KLK6, as specific cleavage at the plasminogen internal bond S460-V461 resulted in the generation of angiostatin, an endogenous inhibitor of angiogenesis and metastatic growth (111). The pro-form and mature form of KLK6 is the only kallikrein, in addition to KLK1, for which the crystal structure has been resolved (31,32). KLK6 is expressed in many tissues (112), with the highest abundance in the central nervous system. KLK6 was detected in an inactive form in human cerebrospinal fluid (113). Crystal structure data suggests that human KLK6 is the functional homologue of rat myelencephalon-specific protease, which is related to the glutamate receptor-mediated cytotoxic injury (32). Investigations on the potential role of KLK6 in central nervous system demyelination showed that myelin basic protein and myelin oligodendrocyte glycoprotein could be the substrates of KLK6 (32). KLK6 was detected in brain tissues of Alzheimer's disease and Parkinson's disease patients (114). Furthermore, several studies have found that the brain of Alzheimer's disease patients contains significantly less KLK6 compared to non-affected individuals, which implies a connection between KLK6 and the pathogenesis of Alzheimer's disease (115,116). KLK6 is also being evaluated as a possible tumor marker in ovarian cancer (117), uterine cancer (118), gastric cancer (119) and colorectal cancer (120).

KLK7

KLK7 was initially cloned as the gene encoding human stratum corneum chymotryptic enzyme (SCCE) (29), and later identified as a member of the kallikrein family (121). Recombinant KLK7 was partly inhibited by the serine protease inhibitor LEKT1 (102), suggesting that it functions as an active serine protease. KLK7 has its physiological role in desquamation, and is known to participate in the cell shedding process (122). Together with KLK5, KLK7 can cleave some dermosomal proteins, and may also play roles in other skin pathophysiologies, including keratinization (123), inflammation by activating interleukin 1β (124), and psoriasis lesions (125). KLK7 may be abnormally expressed in skin where epidermal cell kinetics are disrupted due to inherited and acquired defects, such as Netherton's syndrome, congenital ichthyosiform erythroderma, ichthyosis vulgaris, actinic keratosis, squamous cell carcinoma *in situ*, and invasive squamous cell carcinoma (126). Similar to other members of the KLK family, KLK7 expression has also been investigated in several kinds of malignancies. For example in ovarian cancer, higher KLK7 expression is associated with poorer prognosis (127). KLK7 was also found to be over-expressed in breast cancer with an unfavorable prognosis (128) as well as in cervical cancer and uterine cancer (129,130).

KLK8

Recombinant KLK8 has a trypsin-like activity with a strong preference for Arg over Lys in the P1 position, and its activity was inhibited by typical serine protease inhibitors (131). KLK8 can degrade casein, fibronectin, gelatin, collagen type IV, fibrinogen, and high-molecular-weight kininogen, and also convert human single-chain tissue-type plasminogen activator (65 kDa) to its two-chain form (32 and 33 kDa) by specifically cleaving the peptide bond Arg275-Ile276. These data suggests that the KLK8 protein may be implicated in ECM protein degradation in the area surrounding KLK8-producing cells (131). KLK8 is expressed in mouse hippocample pyramidal neurons involved in hippocampal plasticity (132) and has been suggested to be associated with synaptogenesis and neural development (133,134), as well as in learning and memory (135). KLK8 was found to be ten-fold increased in the hippocampus of Alzheimer's disease patients compared to controls (136) suggesting that it could be a key protein controlling pathogenic events in the hippocampus and may be associated with epilepsy (132). KLK8 was also up-regulated during central nervous system injury (137) and may be a favorable prognosis factor for ovarian cancers (138,139).

KLK9 – KLK15

KLK9 (initially identified as *KLK-L3*) is predicted to encode a serine protease with chymotrypsin-like activity , while the *KLK10* gene (also known as the normal epithelial cell-specific 1 gene (*NES1*)) encodes a secreted serine protease predicted to have trypsin-like enzymatic activity (140). Both genes are regulated by steroid hormones in cancer cell lines, and may have potential prognostic value in hormone dependent cancers (141). *KLK11* was initially identified from hippocampus cDNA (142), and named trypsin-like serine protease and hippostatin (hippocampus and prostate trypsin) due to its similarity to trypsin and its high expression in the prostate. The seminal plasma levels of KLK11 are relatively high (10-15 μg/ml), and purified KLK11 can therefore be isolated by immunoaffinity chromatography (143). KLK11 possesses trypsin-like activity and cleaves synthetic peptides after arginine but not lysine residues, and it does not cleave chymotrypsin substrates (143). The *KLK12* gene gives rise to three splice variants, which is expressed in a variety of tissues (144). Recombinant

KLK12 was found to have serine protease activity demonstrated by cleavage of a chromogenic substrate (H-D-Pro-L-Phe-L-Arg-p-nitroaniline dihydrochloride) (145). As many of the other members of the KLK family, *KLK13* (initially named *KLK-L4*) was identified by positional candidate gene approach in regions around the kallikrein locus (146). The identification of five new *KLK13* splice variants was described, which were not expressed in any other tissue except the human testis (146,147). The recombinant protein interacts and forms complexes with serum protease inhibitors, including α_2-macroglobulin, α_1-antichymotrypsin and α_2-antiplasmin (148,149). The mature KLK13 displays trypsin-like activity with restricted specificity on synthetic and protein substrates, and is capable of autoinactivation *in vitro* (150). Using Phage-display substrate technology, recombinant KLK14 was found to have both trypsin- and chymotrypsin-like activity, with a preference for cleavage after arginine residues (151). Similar to PSA, but unlike other trypsin-like serine proteases, KLK15 does not have an aspartate residue in the substrate-binding pocket, suggesting a chymotrypsin-like substrate specificity (152). The encoded protein can activate pro-PSA (74). KLK9 -KLK15 mostly exhibit a multi-tissue localization pattern and hormone regulated expression (for review see e.g. (11)). To date, there is no known biological function for KLK9 - KLK15; however, their expression has been associated with cancers, mostly in relation to tumor grade, stage or prognosis.

Kallikrein 4 – a unique member of the kallikrein family?

The *KLK4* gene has the typical kallikrein gene structure, with five exons and four introns (87,88). Initial computer analysis of the gene predicted a transcript encoded by all five exons, which would be translated into a pro-KLK4 of 254 amino acids, with a 26-aa signal peptide that would result in an active protein of 224 amino acids after cleavage of the pro-piece. However, extensive screening of cDNA libraries and RACE analysis did not permit the cloning of a 5'-extension deduced to encode the first exon (90). By the use of reverse transcriptase (RT) and PCR of mRNA from the prostate cancer cell line LNCaP and an androgen-dependent prostate cancer xenograft, *KLK4* was found to be the first member of the kallikrein family that has only four coding exons (90). A more detailed analysis of the 5'-end of the *KLK4* mRNA revealed that the transcript with the predicted exon 1 is present only at extremely low levels, while a truncated transcript lacking the putative exon 1 is the physiologically relevant form of *KLK4* mRNA (23). This transcript would thus give rise to a protein lacking the signal peptide and the pro-piece predicted to be encoded by exon 1, and thus be intracellularly localized. This was indeed what was found by immunofluorescence microscopy and biochemical fractionation experiments which demonstrated that KLK4 was a predominantly nuclear protein (23). This was the first demonstration of a different subcellular localization for a KLK other than a secreted form, implying also unique functional role, compared with the other members of the KLK family. Recent independent studies have confirmed the presence of a *KLK4* transcript lacking the putative exon 1 which encoded a nuclear form of the KLK4 protein (91). It is possible that some forms of other KLKs are also intracellularly localized and may therefore have unexpected biological activities.

Conclusions

The lightning expansion of the human kallikrein family from 3 to 15 members during the last decade has generated intense interest for this family of possibly very important proteins. As the genomic era of the kallikrein research is seeing its end, the main focus now is on the delineation of their function and on their potential involvement in a variety of human diseases. Given their hormonal regulation and in some cases tissue restricted expression patterns, an area of major effort has been on their potential role as diagnostic and prognostic biomarkers for cancer for which some KLKs look quite promising, following the example of KLK3 (PSA). However, their potential role in the development of the various cancers has not been well studied, and their exact role in normal and disease states are still largely unknown. In light of these findings and to elucidate KLK function, there is also an intense effort for identifying substrates, as well as inhibitors of KLKs. Continuing research in this area will eventually uncover the real function of the KLKs, and also their potential involvement in the development and progression of cancer and other human diseases.

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