Subject Review

Human Tissue Kallikreins: Physiologic Roles and Applications in Cancer

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Abstract

Tissue kallikreins are members of the S1 family (clan SA) of trypsin-like serine proteases and are present in at least six mammalian orders. In humans, tissue kallikreins (hK) are encoded by 15 structurally similar, steroid hormone–regulated genes (*KLK*) that colocalize to chromosome 19q13.4, representing the largest cluster of contiguous protease genes in the entire genome. hKs are widely expressed in diverse tissues and implicated in a range of normal physiologic functions from the regulation of blood pressure and electrolyte balance to tissue remodeling, prohormone processing, neural plasticity, and skin desquamation. Several lines of evidence suggest that hKs may be involved in cascade reactions and that cross-talk may exist with proteases of other catalytic classes. The proteolytic activity of hKs is regulated in several ways including zymogen activation, endogenous inhibitors, such as serpins, and via internal (auto)cleavage leading to inactivation. Dysregulated hK expression is associated with multiple diseases, primarily cancer. As a consequence, many kallikreins, in addition to hK3/PSA, have been identified as promising diagnostic and/or prognostic biomarkers for several cancer types, including ovarian, breast, and prostate. Recent data also suggest that hKs may be causally involved in carcinogenesis, particularly in tumor metastasis and invasion, and, thus, may represent attractive drug targets to consider for therapeutic intervention. (Mol Cancer Res 2004;2(5):257–80)

Introduction

Proteases/peptidases, defined as enzymes that catalyze peptide bond hydrolysis, perform fundamental functions in all living organisms (1, 2). The "degradome" or complete set of proteases expressed at a given time within a cell, tissue, or organism (3) comprises ~2% of all genes in many organisms. The human genome contains at least 553 protease genes and counting (4). Protease action is always irreversible and can involve either indiscriminant and non-specific degradation of protein substrates, as in apoptosis, or highly specific proteolytic processing or limited hydrolysis of selected target proteins, resulting in a functional change, as in prohormone activation. Proteases are classified according to three major criteria: location of the scissile peptide bond within the substrate (terminal or internal), catalytic mechanism, and evolutionary relationships, as revealed by structure. On the basis of the first criterion, proteases are broadly categorized as either exo- or endopeptidases, respectively. According to the second criterion, endopeptidases are divided into the well-known cysteine, serine, threonine, aspartic, and metalloprotease subgroups. Consistent with the third criterion, proteases of each catalytic class are clustered into several "clans," which in turn include many "families" containing proteases that share significant sequence similarities (5, 6).

Serine proteases were among the first enzymes to be studied extensively (7). Their structural characteristics, catalytic mechanism, and roles in normal physiologic processes (e.g., digestion, coagulation, and cellular and humoral immunity) and in the pathology of many diseases (e.g., cancer, neurodegenerative disorders) have been previously reviewed (5, 8-12). With the exception of a small class of membrane-bound serine proteases, the vast majority are secreted. Furthermore, serine proteases have been organized into 11 evolutionary clans, most of which reside in clan SA of trypsin/chymotrypsin-like serine proteases (6).

Tissue kallikreins (EC 3.4.21) form a subgroup of secreted serine proteases within the S1 family of clan SA. To date, tissue kallikreins have been identified in a variety of species from six mammalian orders including

1. Primates (e.g., human, chimpanzee, baboon, cynomolgus monkey, rhesus monkey, orangutan, gorilla);
2. Rodentia (e.g., mouse, rat, guinea pig, mastomys);
3. Carnivora (e.g., dog, cat);
4. Proboscidea (e.g., elephant);
5. Perissodactyla (e.g., horse); and
6. Artiodactyla (e.g., pig, cow) (13-15).

The number of kallikreins varies among species from 2 in the dog (16) to more than 25 in rodents (15, 17). For a more thorough discussion of kallikreins in non-human species, please refer to our recent review (14). In humans, the tissue kallikrein (hK) family consists of 15 structurally homologous serine

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protease genes that colocalize in tandem to 19q13.4 (18). Of the ~176 serine protease genes within the human genome, this family represents the largest contiguous cluster (4, 19). In fact, the kallikrein gene family is the largest cluster of protease genes of any catalytic class (4, 19).

The discovery of the complete hK family can be divided into two eras. The first era (1930s to 1980s) witnessed the discovery of the "classical" kallikreins. Although originally found in human urine (20, 21), human kallikrein 1 was subsequently identified at abundant levels in the pancreas (in Greek, the "kallikreas"), from which its name was derived (22). However, the gene for this kallikrein, now called *KLK1*, was not discovered until 1985 (23, 24). During the late 1980s, two genes with high structural similarity to *KLK1*, currently known as *KLK2* and *KLK3/PSA*, were cloned and colocalized to the same chromosomal region (19q13.4) with *KLK1* (25-27). At this time, it was concluded that the human kallikrein family had only three members, a statement that would hold for ~10 years.

The second era (1994 to 2001) saw the expansion of the kallikrein family to 15 genes and the complete description of the human kallikrein locus. During the mid- to late 1990s, independent researchers cloned several novel serine protease genes with significant similarities to the classical kallikrein genes. These included, *human stratum corneum chymotryptic enzyme* (*HSCCE*)/*KLK7* (28), *normal epithelial cell-specific gene 1* (*NES1*)/*KLK10* (29), *protease M/zyme/neurosin/KLK6* (30-32), *neuropsin/TADG-14/KLK8* (33, 34), and *trypsin-like serine protease* (*TLSP*)/*hippostasin/KLK11* (35, 36) These genes were subsequently recognized as tissue kallikrein genes and mapped to 19q13.4 by Diamandis and colleagues (37-43). An additional seven kallikrein genes were independently cloned by our group and others, namely: *prostase/KLK-L1/KLK4/ARM1/PRSS17* (44-46), *human stratum corneum tryptic enzyme* (*HSCTE*)/*KLK-L2/KLK5* (47, 48), *KLK-L3/KLK9* (40), *KLK-L4/KLK13* (49), *KLK-L5/KLK12* (50), *KLK-L6/KLK14* (51, 52), and *prostinogen/KLK15* (53, 54), as well as the first kallikrein pseudogene, *ΨKLK1*.[2] According to the official nomenclature, kallikrein gene and protein symbols are currently denoted "*KLK*" and "hK," respectively (55). Gene numbering starts from centromere to telomere on chromosome 19q13.4 with the exception of the three classical kallikreins, for which the existing nomenclature was retained (56). Table 1 lists all official and alternative kallikrein gene and protein names. [Please note that plasma kallikrein (18, 57, 58) is not a member of the tissue kallikrein family.]

Locus Organization

Thus far, the topology of the *KLK* locus has only been described in detail within the human (38, 59), chimpanzee,[3] mouse (17), and rat (15) genomes (Fig. 1). In general, mammalian *KLK* loci contain a single copy of *KLK4-KLK15* genes and varying numbers of classical *KLK* genes and pseudogenes, all of which presumably arose due to gene duplication events. In humans, the *KLK* locus spans ~300 kb on the long arm of chromosome 19 in cytogenic region 13.3 to 13.4 and is bound centromerically by the testicular acid phosphatase gene (*ACPT*; ref. 60), and telomerically by a cancer-associated gene (*CAG*)[4] and *Siglec-9*, a member of the sialic acid-binding Ig-like lectin (Siglec) family (61) (Fig. 1A). The *KLK* genes are tightly clustered in a tandem array, and are not intervened by any non-*KLK* genes. The three classical human *KLK* genes (*KLK1*, *KLK2*, and *KLK3*) are clustered within a 60-kb region with *KLK15*, whereas *KLK4-KLK14* and the *ΨKLK1* pseudogene are all located telomeric to *KLK2*. The direction of transcription of all genes is from telomere to centromere with the exception of *KLK3* and *KLK2*.

The syntenic relationship of *KLK* gene organization is relatively conserved among human, chimpanzee, and rodent genomes. The chimpanzee *KLK* locus is strikingly similar to the human locus, spans ~350 kb of genomic sequence on chromosome 20, and contains orthologs to all 15 human *KLK* genes, which share more than 99% sequence similarity at the DNA and amino acid levels (Fig. 1B). Although the relative location and direction of transcription of *KLK1* and *KLK4-KLK14* is conserved among the human, chimpanzee, mouse, and rat loci, discrepancies exist with respect to locus size and number of *KLK1* and *KLK15* paralogs and pseudogenes within the mouse and rat genomes, due to additional gene duplication events. In the mouse, the *KLK* locus covers 590 kb within cytogenic region B2 on chromosome 7 and comprises 26 genes and 11 pseudogenes (Fig. 1C). In contrast to the human and chimp, the mouse lacks *KLK2* and *KLK3* orthologs but contains 23 *KLK1* paralogs, 14 of which are functional. The latter reside between *KLK1* and *KLK15* in a 290-kb region, an area that is only 1.5 kb in length within the human locus. Generally, mouse and human *KLK* orthologs share ~77% to 80% sequence similarity (62). The rat *KLK* locus spans 580 kb within cytogenic region q21 on chromosome 1, contains 22 genes and 19 pseudogenes, and is also devoid of *KLK2* and *KLK3* orthologs (Fig. 1D). Interestingly, this locus contains nine duplications of a ~30-kb region harboring the *KLK1*, *KLK15*, and *ΨKLK2* genes between *ΨKLK2* and *KLK4*, resulting in nine paralogs of each gene. However, only the *KLK1* paralogs are functional. Rat *KLK* genes share ~80% to 85% sequence similarity with their human orthologs.

Phylogenetic analyses indicate that the classical *KLKs* of the human, mouse, and rat represent a distinct monophyletic group within the kallikrein family, separate from the more recently discovered kallikreins, *KLK4-KLK15* (15, 17, 54, 59, 63). Moreover, within the classical *KLK* branch itself, *KLK1* genes of the human, mouse, and rat form separate, species-specific subgroups, in contrast to rodent *ΨKLK2* genes and human *KLK2* and *KLK3* that cluster into one subgroup. The latter suggests that these genes shared a common ancestral *KLK2* gene that was subsequently silenced in rodents and evolved into *KLK2* and *KLK3* in primates (15). Taken together, the

[2] Our data submitted for publication.
[3] Our data submitted for publication.
[4] Our data submitted for publication.

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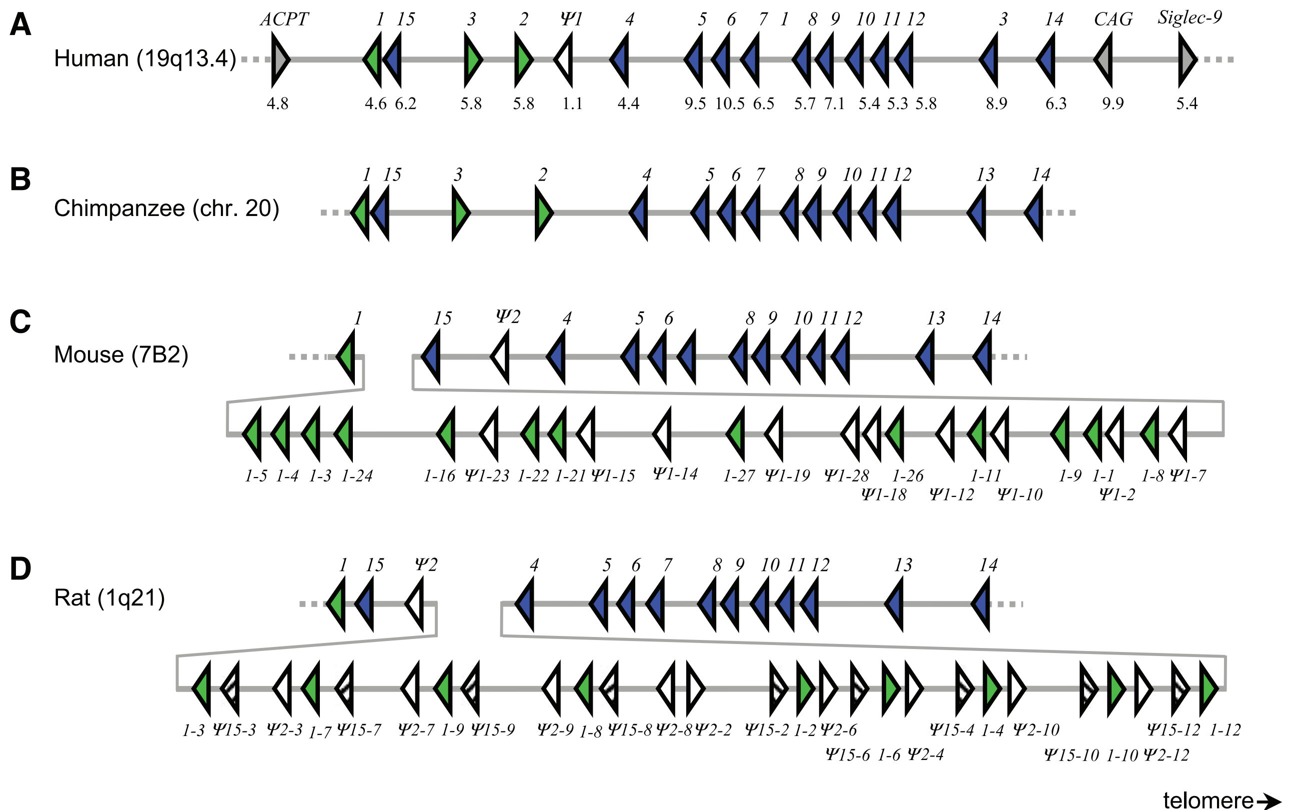

FIGURE 1. Organization of the tissue kallikrein gene loci in human (**A**), chimpanzee (**B**), mouse (**C**), and rat (**D**) genomes. *Arrowheads* indicate the location of genes and their direction of transcription. *Green arrowheads*, classical glandular kallikrein genes (*KLK1*, *KLK2*, and *KLK3*) and mouse and rat *KLK1* paralogs. *Blue arrowheads*, non-classical kallikrein genes, *KLK4 – KLK15*. Kallikrein pseudogenes are represented by *white arrowheads* with the exception of rat *KLK15* paralogous pseudogenes that are shown as *striped white arrowheads*. *Grey arrowheads*, non-kallikrein genes (*ACPT*, *CAG*, and *Siglec-9*). Official gene names are abbreviated to their numbers and indicated above each *arrowhead*. The nomenclature proposed by Olsson et al. (15) for paralogs of the mouse and rat loci is beneath each *arrowhead*. **A.** Gene lengths are indicated below each gene in the human kallikrein locus only. Figure is not drawn to scale. Modified from refs. (15, 17, 18) and our unpublished data (for chimpanzee).

above evidence implies that the classical *KLKs* likely evolved independently in the human, mouse, and rat after the divergence of the lineages, in contrast to *KLK4-KLK15* that probably diverged before their split. However, it is still not clear whether the classical *KLKs* have evolved from the *KLK4-KLK15* group or if the two groups are monophyletic and share a common ancestor. In any event, the strong conservation of *KLK4-KLK15* in these species suggests that the encoded serine proteases perform essential functions in mammals. In contrast, the ''late-evolving'' classical *KLKs* may encode proteins possessing functions that are rather unique to the primate and rodent orders (15).

Table 1. Official and Alternative Kallikrein Gene and Protein Names

Official Gene/Protein	Other Names/Symbols	GenBank Accessions	References
KLK1/hK1	Tissue/pancreatic/renal/urinary kallikrein, hPRK	M25629, M33105	(24, 359)
KLK2/hK2	Human glandular kallikrein 1, hGK-1	M18157	(25)
KLK3/hK3	Prostate-specific antigen, PSA, APS	X14810, M24543, M27274	(26, 360 – 362)
KLK4/hK4	Prostase, *KLK-L1*, EMSP1, PRSS17, ARM1	AF113141	(44 – 46, 100, 229)
KLK5/hK5	*KLK-L2*, HSCTE	AF135028	(47 – 48)
KLK6/hK6	Zyme, Protease M, Neurosin, PRSS9	D78203 (mRNA), AF149289 (Full gene)	(30 – 32, 41)
KLK7/hK7	HSCCE, PRSS6	L33404 (mRNA), AF166330 (Full gene)	(28, 42)
KLK8/hK8	Neuropsin, Ovasin, *TADG-14*, PRSS19, HNP	AB009849	(33, 34)
KLK9/hK9	*KLK-L3*	AF135026	(40)
KLK10/hK10	*NES1*, PRSSL1	NM_002776 (mRNA), AF055481 (Full gene)	(29, 39)
KLK11/hK11	TLSP/Hippostasin, PRSS20	AB012917 (mRNA), AF164623 (Full gene)	(35, 43)
KLK12/hK12	*KLK-L5*	AF135025	(50)
KLK13/hK13	*KLK-L4*	AF135024	(49)
KLK14/hK14	*KLK-L6*	AF161221	(52)
KLK15/hK15	Prostinogen, HSRNASPH	AF242195	(53, 54)

NOTE: Adapted from ref. (18) with permission from copyright owners.

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Structural Features of Kallikrein Genes and Proteins

Members of the human kallikrein gene family generally share 30% to 50% similarity at the DNA and amino acid levels, exclusive of *KLK2* and *KLK3* that possess 80% similarity and are, thus, considered the most closely related *KLKs* (18). As expected, *KLK* genes and the encoded hK proteins share several defining structural characteristics, illustrated and listed in Fig. 2. All *KLK* genes colocalize to the same chromosomal region (19q13.4) and code for serine proteases. *KLK* genes typically range from 4 to 10 kb in length and their organization, in terms of number and length of coding exons, intronic phase pattern, and location of start, stop and catalytic histidine, aspartate, and serine codons, is remarkably similar (18) (Fig. 2A). Of the few structural differences that have been found, all occur within non-coding regions. For instance, intron length is variable among *KLK* genes, leading to different gene lengths. Consensus GT-AG splice junctions are conserved among all *KLKs*, with the exception of the *KLK10* gene that possesses a GC-AG splice site pair within intron 4 (39). The majority of the recently discovered *KLKs* (*KLK4-KLK15*) possess one or two non-coding exons within the 5′ untranslated region (UTR), whereas the classical *KLKs* (*KLK1*, *KLK2*, and *KLK3*) do not. Furthermore, the 3′ UTR beyond the stop codon is quite variable in length among *KLKs* and contains either a consensus (AATAAA) or variant polyadenylation signal ~15 bp from the polyadenylic acid tail (18).

Kallikrein proteins are single-chain secreted serine proteases translated as preproenzymes (Fig. 2B). Each contains a signal peptide of 16 to 30 amino acids at their NH_2 terminus, followed by a pro-peptide of four to nine amino acids, and catalytic domain, which comprises the mature, enzymatically active, protein (18). Pro- and mature enzyme forms result from the sequential cleavage of the signal and pro-peptides on entry into the secretory pathway and on activation, respectively. It is important to note, however, that the majority of these cleavage sites are predicted; only a few have been experimentally verified (refs. 28, 36, 47, 64 and our unpublished data).

The calculated molecular weight of the peptide moiety of pro-hK proteins ranges from ~23,000 to 26,000 (18). However, due to glycosylation, greater masses have been observed for several kallikreins including native hK1 (65), hK3 (66), hK5 (47), and hK7 (28) as well as recombinant hK2 (67) hK5 (47, 68), hK6 (69), hK7 (28, 70), and hK13 (71). (It is important to note, however, that the presence, absence, or type of glycosylation found in recombinant hKs produced in heterologous expression systems may not accurately reflect the status and nature of glycosylation of the native protein.) With the exception of hK1, in which O-linked glycosylation has been observed (65), all other reported glycosylation events, thus far, involve the addition of N-linked carbohydrates. Furthermore, glycosylation site prediction programs (72) on the Center for Biological Sequence Analysis website (http://www.cbs.dtu.dk)

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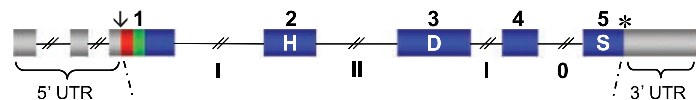
A General *KLK* gene characteristics

- Co-localize to 19q13.4
- encode serine proteases
- 5 coding exons and 4 intervening introns with conserved intron phase pattern (I, II, I, 0)
- coding exon lengths are similar or identical (see Ref. 18)
- start codon is located 8-87bp from the end of coding exon 1
- stop codon is found ~150-189 bp from the start of coding exon 5 catalytic triad codon positions are conserved (H codon near the end of coding exon 2, D in the middle of coding exon 3 and S near the start of coding exon 5)
- one or two 5' UTR exons in most *KLKs* (not classical *KLKs*)
- variable 3' UTR length
- more than one mRNA transcript/gene
- steroid hormone regulated

B hK protein characteristics

- single-chain preproenzymes consisting of:
  - signal peptide of 16-33 aa
  - pro-peptide of 4-9 aa (except hK5 -37aa)
  - mature, enzymatically active enzyme of 227-252 aa
  - conserved catalytic triad (H,D,S)
  - S1 amino acid found 6 residues before catalytic S
  - 10-12 conserved cysteine residues form five (in hK1-3 and hK13) or six (the rest) disulfide bonds



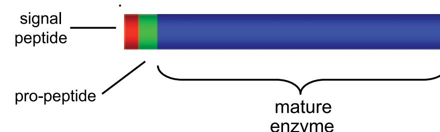


FIGURE 2. Common structural features and schematic representation of a typical kallikrein gene (**A**) and protein (**B**). **A.** *boxes*, exons; *lines*, intervening intron. Coding exons are shown in *red*, *green*, and *blue*. *Shaded boxes*, untranslated exons and regions. The *numbers above the exons* indicate coding exon number and the *Roman numerals* below, the intron phase. Coding exon 1 harbors the start codon (indicated by ↓) and codes for the signal (*red*) and propeptides (*green*). Coding exons 2, 3, and 5 contain the histidine (*H*), aspartic acid (*D*), and serine (*S*) codons of the catalytic triad. Coding exon 5 harbors the stop codon (*). **B.** *red box*, signal peptide; *green box*, pro-peptide; *blue box*, mature, enzymatically active protein. Pro- and mature enzyme forms result from the sequential cleavage of the signal and pro-peptides on entry into the secretory pathway and on activation, respectively. It is important to note that the majority of these cleavage sites are predicted; only a few have been experimentally verified. Figure is not drawn to scale.

Table 2. Specificity, Physiologic Substrates, and Post-translation Regulation of Human Kallikrein Proteins

hK	Pro-peptide Cleavage Site*	S1 aa	Specificity	P1 Position	Possible Physiologic Substrates	Inhibitors	Auto Activation	Activation by Other hK	Auto-/or Degradation
hK1	R↓I[8]	Asp	Trypsin-like	Arg, Met (363), Phe (364-366)	Low MW kininogen (367), preANF (368), pro-insulin, LLP, prorenin, VIP, procollagenase, angiotensinogen (reviewed in ref. 191), BK B2 (369)	Kallistatin (187, 188), PCI (370, 371), AAT (372, 373), placental bikunin (374)			
hK2	R↓I[8]	Asp	Trypsin-like	Arg (174, 365, 375)	Seminogelin I/II (174, 204), IGFBP-3 (376), pro-uPA (243), fibronectin (204)	PCI (174, 377), PI-6 (378), PAI-1 (379), ATIII (380), α_2AP (381), ACT (185, 381), α_2M (185, 377)	✓ (200, 201)		✓ (173, 200)
hK3	R↓I[8]	Ser	Chymotrypsin-like	Leu, Phe (382), Tyr (203)	Seminogelin I/II, fibronectin (205, 233), laminin (233), lysozyme (382), plasminogen (248), IGFBP-3 (383, 384), TGF-β (294), PTHrp (385, 386)	ACT (387, 388), α_2M (387), PCI (389), AAT (390), ATIII (380)		hK2 (173, 202, 203), hK4 (242)[†], hK15 (53)	✓ (391)[†]
hK4	Q↓I[5]	Asp	Trypsin-like	Arg (242)[†,§], Lys (242)[†]	pro-uPA, PAP (242)[†]		✓ (242)[†]		
hK5	R↓I[38]	Asp	Trypsin-like	Arg > Lys[§]	corneodesmosin (214), ECM, fibrinogen[§]	α_2AP, ATIII, α_2M[§]			
hK6	K↓L[6]	Asp	Trypsin-like	Arg > Lys (69, 392)	ECM, fibrinogen, APP (178)[‖], fibronectin, laminin (69), plasminogen[¶]	ATIII, α_2AP, AAT (178), ACT (178, 393)	✓ (31, 178)[‖]		✓ (69, 178)
hK7	K↓I[8]	Asn	Chymotrypsin-like	Tyr (28)	IL-1β (218), corneodesmosin (214)				✓ (28)
hK8	K↓V[5]	Asp	Trypsin-like	Arg[§]	MBP (394)				
hK9	R↓A[4]	Gly	Chymotrypsin-like						
hK10	R↓L[10]	Asp	Trypsin-like						
hK11	R↓I[4]	Asp	Trypsin-like	Arg (36)[§]					
hK12	K↓I[5]	Asp	Trypsin-like						
hK13	K↓V[6]	Asp	Trypsin-like	Arg > Lys[§]	ECM[§], plasminogen (179)	α_2M, α_2AP, ACT (71)	✓ (179)		✓ (179)
hK14	K↓I[7]	Asp	Trypsin-like	Arg > Lys[§]	ECM[§]				✓[§]
hK15	K↓L[6]	Glu	Trypsin-like	Arg (53), Lys (53, 175)					

Abbreviations: AAT, α_1-antitrypsin; α_2AP, α_2-antiplasmin; APP, amyloid precursor protein; ATIII, antithrombin III; BK B2, human bradykinin B2 receptor; LLP, low density lipoprotein; MBP, myelin basic protein; PAI-1, plasminogen activator inhibitor-2; PAP, prostatic acid phosphatase; PCI, protein C inhibitor; PI-6, protease inhibitor-6; preANF, precursor of atrial natriuretic factor; pro-uPA, pro-form of urokinase-type plasminogen activator; PTHrp, parathyroid hormone-related peptide; VIP, vasoactive intestinal peptide.

*Arrows indicate the cleavage site and pro-hK numbering is shown.

† A chimeric form of hK4 (ch-hK4) was used.

‡ Degradation in vivo by unknown proteins.

§ Our unpublished data.

‖ Our data submitted for publication.

¶ G. Sotiropoulou, personal communication.

indicate that most hK proteins harbor one or more putative N-glycosylation sites or sequons, Asn-*X*-Ser/Thr (in which *X* is any amino acid except Pro; ref. 73), whereas only a few kallikreins have potential Ser/Thr residues involved in O-linked glycosylation. Collectively, experimental and bioinformatic data suggest that most, if not all, kallikreins are glycoproteins in vivo. Glycosylation of many proteins is important for their proper expression and function (74-77).

The amino acid of the S1 binding pocket, primarily responsible for substrate specificity in serine proteases (8), is found six amino acids NH_2-terminal of the catalytic serine residue in all kallikrein enzymes at position 189, according to chymotrypsin numbering (78). Multiple alignments of deduced protein sequences indicate that 12 kallikreins possess an aspartate or glutamate residue in this position and are expected to cleave on the carboxyl side of basic amino acids such as arginine or lysine, similar to trypsin. In contrast, the remaining three, hK3, hK7, and hK9, have non-polar serine, asparagine, and glycine residues, respectively, conferring a chymotrypsin-like specificity (18) (Table 2). Thus far, experimental verification of substrate specificity has been done for all kallikrein enzymes with the exception of hK9, hK10, and hK12 (Table 2).

To date, X-ray crystallographic structures have been resolved for two human kallikreins, namely mature hK1 (79)

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and mature and pro-hK6 (64, 69), as well as for several non-human kallikreins, such as horse prostate kallikrein, an hK3 ortholog (80), mouse neuropsin/hK8 (81), mouse glandular kallikrein-13 (82), and porcine pancreatic kallikrein A (83). As members of the S1 family (clan SA) of serine proteases, kallikreins possess the archetypal tertiary structure of trypsin/chymotrypsin-like serine peptidases (6), which consist of two juxtaposed six-stranded anti-parallel β-barrels and two α-helices, with the active site [His^{57}, Asp^{102}, and Ser^{195}, chymotrypsin numbering (78)] bridging the barrels (84, 85). The stereo ribbon plot for pro-hK6 is shown in Fig. 3.

Structural heterogeneity among kallikrein enzymes can be attributed to the variable external surface loops surrounding the substrate-binding site, which are known to control their activity, define substrate and inhibitor specificity, and function in autolytic regulation (8, 69, 86-89). Depending on the hK in question, either five or six disulfide bonds serve to covalently link the polypeptide chain and provide structural rigidity to the surface loops surrounding the substrate-binding site. For instance, the classical kallikreins possess a unique surface loop named the "kallikrein loop," not present in its entirety in any other kallikrein and absent in other serine proteases. Glycosylation of the kallikrein loop, and others, may serve to regulate kallikrein activity. For example, N-linked oligosaccharides present on the kallikrein loop determine the size of the S2 pocket and affect the P2 specificity of recombinant mouse hK8 (87). As well, the kallikrein loop, along with another surface loop, may be required for the regulated secretion of mouse hK8 (87). Within horse prostate kallikrein (hK3 ortholog), the kallikrein loop, seems to have a direct role in enzymatic control and substrate selectivity, because it protrudes over the catalytic region, blocking the entrance to the S1 specificity pocket (80). Furthermore, hK15 is unique in that it possesses an eight-amino-acid surface loop not present in any other kallikrein protein (54).

Alternative Messenger RNA Transcripts

In the post-genomic era, with the discovery of an unexpectedly low number of genes (~32,000) within the human genome sequence (90, 91), it has become clear that the generation of protein complexity occurs mainly via expansion of the human transcriptome. One of the major and most well-described mechanisms involved is alternative pre-mRNA splicing, whereby a single primary gene transcript or pre-mRNA gives rise to many mature mRNA transcripts, encoding many structurally and functionally distinct proteins (92). Indeed, recent genome-wide analyses indicate that 35% to 74% of all human genes have at least one alternative splice form (93, 94). The use of alternative promoters/transcriptional start sites (95) and polyadenylation signals (96) comprise additional sources for increasing the informational content of the genome.

Alternative pre-mRNA splicing, transcriptional start sites, and polyadenylation signals are common events among members of the kallikrein gene family. In addition to their classical mRNA forms, each kallikrein gene possesses at least one alternative transcript. In fact, a total of 70 alternative *KLK* mRNA isoforms have been identified to date, exclusive of the classical form (Table 3). Thus, a new dimension to the *KLK* family exists. With respect to alternative splicing events among *KLK* genes, the majority occur in coding regions and primarily involve exon skipping, followed by exon extension/truncation and intron retention, with only a few events occurring within the 5′ UTR. Consensus GT-AG splice sites are conserved in almost all kallikrein splice variants, with a few exceptions. For instance, a GC-AG splice site pair is present in a *KLK5* variant with alternative splicing in the 5′ UTR (GenBank accession no. AY279381). A TG-AG splice junction is found in a *KLK15* variant, in which coding exon 3 is lengthened and exon 4 is excluded (GenBank accession no. AY373374) and CC-AG pairs are found in several *KLK3* variants (97). Recognition of

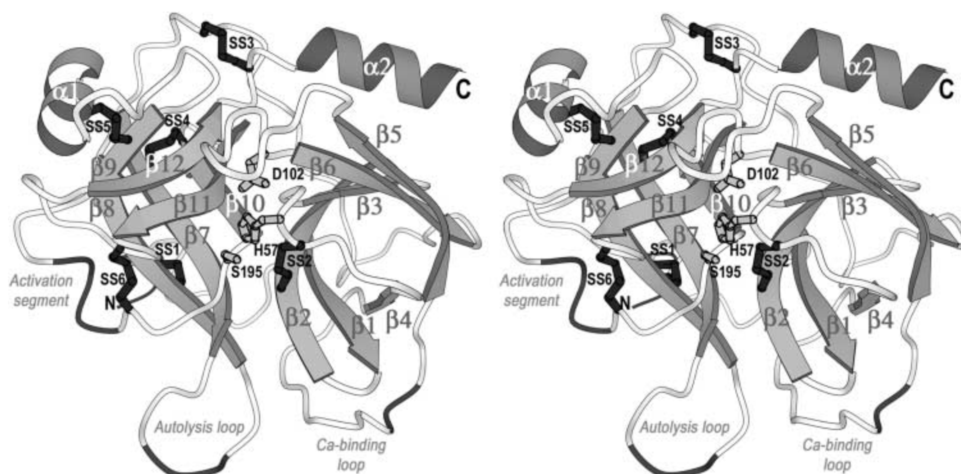

FIGURE 3. Crystal structure of pro-hK6 as solved by Gomis-Ruth et al. (64). Stereo ribbon plot of pro-hK6 shown in the traditional serine proteinase standard orientation (358) (i.e., looking into the active site cleft). The regular secondary structure elements are displayed as *arrows* (β*-strands*) and *ribbons* (α*-helices*) and labeled (β*1*-β*12* and α*1*-α*2*). The side chains of the residues of the catalytic triad (*light gray*) and the six disulfide bonds (*dark gray*; *SS1-SS6*) are also shown as *stick models* and labeled. The NH_2 and COOH termini and the positions of characteristic structural loops (i.e., autolysis loop, Ca-binding loop) are also indicated. (With *dark gray coils* are shown the poorly defined and undefined main-chain stretches.) Adapted with permission from ref. 64.

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Table 3. Reported Human Kallikrein Variant Messenger RNA Transcripts

Kallikrein	Reported No. of Variant Transcripts*	GenBank Accession No.	References
KLK1	3	NS[†] (two variants), AY429508	refs. 395, 396 and our unpublished data
KLK2	6	NS, AF188747, AF188746, AF188745, AY429510, AY429509	refs. 103, 139 and our unpublished data
KLK3	11	AJ459783, AF335477, AF335478, AJ512346, AJ459784, M21896, AJ459782, AJ310937/M21897, AJ310938, NM_145864, NS	(26, 97, 104, 105, 132, 397)
KLK4	8	AF148532 (two variants), NS (two variants), AF228497, AF259964, AF259971, AF259970	(45, 100, 229, 398)
KLK5	5	AY461805, AY279381, AY279380, AF435980, AF435981	ref. 101 and our unpublished data
KLK6	6	AY279383, AY318867, AY318869, AY318870, AY318868, AY457039	our unpublished data
KLK7	3	NM_19277, AF411215, AF411214	(42, 101)
KLK8	4	NM_144505, NM_144506, NM_144506, BC040877	(102, 267, 399)
KLK9	2	NS, AF135026	our unpublished data
KLK10	1	BC002710	(399)
KLK11	3	NM_144947, AB078780, BC022068/NM_006853	(36, 399, 400)
KLK12	3	NM_019598, NM_145895, AY358524	(50, 401)
KLK13	8	NS (five variants), AB108823, AB108824, AL050220	(108, 133)
KLK14	2	NS	(51)
KLK15	5	AF242193 (three variants), AY373373, AY373374	ref. 54 and our unpublished data

*All mRNA transcripts (including splice variants, transcripts with alternative transcriptional start sites, and polyadenylation signals and combinations thereof) exclusive of the classical transcript.
[†] Not submitted to GenBank.

these atypical splice sites by the spliceosome is possible in association with a conserved splice site (98, 99). Additional mRNA transcripts with alternative transcriptional start sites have been identified for several kallikrein genes including *KLK3*, *KLK4*, *KLK5*, *KLK6*, *KLK7*, and *KLK11*, and are likely the products of alternative promoters (refs. 26, 100-102 and our data submitted for publication). Furthermore, several transcripts arising from alternative polyadenylation sites exist for *KLK2* (103), *KLK3* (97), and *KLK7* (101). Many *KLK* transcripts exhibit a combination of alternative splicing events coupled with alternative transcription start site and polyadenylic acid signal usage.

By open reading frame analysis, it has been predicted that several alternatively spliced kallikrein transcripts will produce unique protein isoforms mainly due to in-frame usage of alternative translation initiation and termination codons, in-frame insertions or deletions in the middle of the protein sequence, and to a lesser extent, due to frameshifts that introduce premature stop codons. In most cases, the sequence encoding the signal peptide is retained, indicating that most kallikrein protein variants, on successful translation, are likely to be secreted and present in biological fluids, which may have clinical relevance in biomarker development. However, in the case of *KLK4*, one transcript isoform excludes the exon predicted to code for the signal peptide leading to the production of an intracellular protein (100), which may have unique functional implications. In some instances, alternative splicing may compromise the serine protease activity of the kallikrein protein due to exclusion of one or more residues of the conserved catalytic triad (H, D, S). Generally, most of these putative protein isoforms have not been isolated, with the exception of a few proteins encoded by *KLK3* variants (97, 104, 105). Although the protein coding region is unaffected, variations in the 5′ or 3′ UTRs may have an effect on post-transcriptional regulation because these regions are known to be important in post-transcriptional regulation including mRNA stability, localization, and translational activation or repression (106, 107). Additional details on alternative kallikrein transcripts and their predicted proteins can be found in the literature cited in Table 3.

Tissue Expression and Cellular Localization

Kallikreins are expressed in a myriad of tissues at both the mRNA and protein levels. As delineated by Northern blot, reverse transcription-PCR, and ELISA methodologies collectively, each kallikrein displays a relatively broad tissue expression pattern, with highest expression levels within a few major tissues and lower levels of expression in many others (18, 63, 71, 108-116). Interestingly, kallikreins are often coexpressed within the same tissues. The most notable example is the concurrent and almost exclusive expression of *KLK2*, *KLK3*, *KLK4*, *KLK11*, and *KLK15* in the prostate, at the mRNA level. As well, almost every kallikrein is expressed in the salivary gland, while subgroups reside in the skin (*KLK1*, *KLK4*, *KLK5*, *KLK6*, *KLK7*, *KLK8*, *KLK9*, *KLK10*, *KLK11*, *KLK13*, and *KLK14*), breast (*KLK5*, *KLK6*, *KLK10*, *KLK13*), pancreas (*KLK1*, *KLK6-KLK13*), and the central nervous system (*KLK6*, *KLK7*, *KLK8*, *KLK9*, *KLK14*). The presence of kallikreins in biological fluids, such as serum, seminal plasma, and the milk of lactating women, confirms that they are secreted proteins in vivo. The functional implications of kallikrein coexpression are discussed in a later section.

Furthermore, in situ and/or immunohistochemistry studies indicate that kallikreins, including hK3 (117, 118), hK4 (109, 119), hK6 (120-122), hK7 (123), hK9 (124), *KLK10*/hK10 (121, 122, 125), *KLK11*/hK11 (115, 126), hK13 (122, 127), *KLK14*/hK14 (51, 116), are localized predominantly in the cytoplasm of glandular epithelia, from which they are likely secreted. The hK1, hK6, hK10, and hK13 proteins have also been localized to the epithelium of the choroid plexus and other cell types within the central and peripheral nervous systems

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(120, 121, 127, 128). With respect to the skin, hK5 and hK7 expression was found to be restricted to the stratum granulosum of the normal epidermis (129-131). A recent in situ hybridization study indicates that several other *KLKs* are also prominently expressed in the stratum granulosum as well as in the inner root sheath of hair follicular epithelium and the cytoplasm of cells within the eccrine sweat glands and sebaceous glands (108).

Tissue-specific patterns of expression have also been documented for many alternative mRNA transcripts of kallikrein genes. For example, a *KLK2* and *KLK3* splice variant, both with a partially retained intron, are exclusively expressed in the prostatic epithelium (132). Splice variants of *KLK4*, *KLK8*, and *KLK13* gene transcripts were found to be the predominant mRNA species in the skin (108). One variant of the *KLK8* gene is predominately expressed in the pancreas, while another variant is preferentially expressed in adult brain and hippocampus (102). The *KLK11* gene has two tissue-specific mRNA isoforms, known as the brain type and prostate type, the former of which is expressed in the brain and prostate and the latter that is expressed exclusively in the prostate (36). Furthermore, several testis-specific splice variants of *KLK13* have been identified (133). Hooper et al. (51) have discovered a 1.5-kb transcript of *KLK14* transcribed only in the prostate and another 1.9-kb transcript expressed exclusively in skeletal muscle. Furthermore, on transfection of a green fluorescent protein (GFP)-tagged *KLK4* transcript variant, lacking the sequence coding for the signal peptide, into COS and HeLa cells, the encoded protein was predominantly localized in the nucleus (100). The physiologic and clinical relevance of alternative kallikrein mRNA transcripts warrants further investigation.

Regulation of Kallikrein Gene Expression and Protein Function

Transcriptional Control of Gene Expression

Transcriptional regulation of eukaryotic genes is a complex process that requires many basal transcription factors for initiation and promoter-specific regulatory protein(s) (activators or repressors) that either enhance or repress target gene expression depending on the nature of signaling stimuli (134).

The regulation of gene expression by steroid hormones, mediated on binding to their cognate receptors, plays an important role in the normal development and function of many organs as well as in the pathogenesis of endocrine-related cancers (135-138). Numerous in vitro and in vivo studies confirm that all human kallikrein genes are under steroid hormone regulation in endocrine-related tissues and cell lines (41, 42, 44, 48-50, 52, 54, 113, 139-147). The most notable example is the classical up-regulation of *KLK2* and *KLK3* transcription in response to androgens and progestins in prostate and breast cancer cell lines (139, 142, 143). Conversely, other kallikreins such as *KLK1*, *KLK6*, and *KLK10* are more responsive to estrogens (41, 145, 148). An interesting observation is the differential pattern of hormonal regulation of certain genes, for instance, *KLK4* is up-regulated by androgens in prostate and breast cancer cell lines (44, 46) and by estrogens in endometrial cancer cell lines (144) and *KLK12* is up-regulated by androgens and progestins in prostate cancer cell lines and by estrogens and progestins in breast cancer cell lines (50).

Functional characterization of kallikrein gene promoters and enhancers may aid in delineating the mechanism of transcriptional regulation by steroid hormone-receptor complexes. These complexes can modulate transcription of target genes in a direct or indirect fashion (149). In the former, the complex binds directly to *cis*-acting DNA sequences known as hormone response elements (HRE) in the promoter/enhancer regions of regulated genes, thereby recruiting necessary cofactors that interact with the basic transcription machinery to regulate gene expression. In the indirect pathway, hormone-receptor complexes do not bind to cognate hormone response elements and indirectly modulate gene expression via interactions with *trans*-acting transcription factors. Thus far, promoters have only been characterized for *KLK1-3* and *KLK10*.

The *KLK1* promoter harbors a putative estrogen response element (ERE) thought to mediate estrogenic regulation, but has not been functionally tested (150). Several androgen responsive elements (ARE) within the proximal promoter and enhancer regions of *KLK2* and *KLK3* genes have been identified and believed to be primarily responsible for transcriptional regulation by androgens. *KLK2* has two AREs; one at position −170 within its promoter (25, 141) and another in the enhancer region −3819 to −3805 upstream from the transcription start site (151). The *KLK3* proximal promoter harbors two functional AREs (ARE-I and ARE-II) at positions −170 and −400 (140, 152) and an additional ARE (ARE-III) in the far upstream enhancer region (−4,136), which has a dramatic effect on *KLK3* transcription, in comparison to ARE-I and ARE-II (153-156). Furthermore, five additional low-affinity AREs have been identified close to ARE-III (157). Conversely, *KLK10* promoter and enhancer regions do not harbor functional hormone response elements directly involved in mediating the apparent transcriptional regulation by steroid hormone-receptor complexes (146). As is the case for other genes and gene families, active hormone response elements may be located within exons or UTRs of the *KLK10* gene or elsewhere in the kallikrein locus, respectively.

The promoter and enhancer regions of the 11 remaining human kallikrein genes have not as yet been functionally characterized. However, sequence analysis has identified putative AREs in the promoter regions of *KLK4*, *KLK14*, and *KLK15* genes (45, 147, 158).

Accumulating reports indicate that the function of steroid hormone receptors is regulated by many coactivators/repressors that act as bridging molecules between hormone-receptor complexes and the basal transcription machinery to either activate or inhibit transcriptional regulation (159). For instance, the relative levels of several coactivators/repressors might differentially modulate the transcriptional activity within the promoter/enhancer region of *KLK2* and *KLK3* of various breast cancer cell lines (160).

Furthermore, several recent studies point to the possibility of cross-talk between steroid hormone signaling with other signal transduction pathways in the regulation of kallikrein gene transcription. For instance, Sadar (161) suggests that cross-talk

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between androgen receptor (AR) and protein kinase A signal transduction pathways contributes to the androgen-independent induction of *KLK3* gene expression. Transcription factors activator protein and a Fos-containing protein complex distinct from activator protein were also reported to regulate *KLK2* and *KLK3* gene transcription (162, 163). As well, the *KLK10* promoter was found to harbor potential AP1-binding, SP1-binding, and adenosine 3′,5′-monophosphate responsive element sequences (164). Wang et al. (165) have discovered that a novel GAGATA transcription factor binds to a *cis*-regulatory element located within the enhancer region of the *KLK3* promoter and is required for the maximum transcriptional response to androgens. Conversely, a negative regulatory *cis*-element named XBE was identified within the *KLK3* enhancer region, and was found to recruit both the AR and the p65 subunit of nuclear factor (NF)-κB AR, leading to the down-regulation of AR-mediated transcription of *KLK3* (166). Thus, cross-talk exists between AR and NF-κB p65 transcription factors and was found to occur via novel mechanism through which the factors compete for binding at a common DNA element.

Moreover, epigenetic control of gene expression such as DNA methylation may also be implicated in regulation of kallikrein gene transcription, particularly during carcinogenesis. The dramatic-down regulation of the *KLK10* gene in breast cancer and in acute lymphoblastic leukemia has been attributed primarily to hypermethylation of exon 3 with this gene (167, 168). This mechanism is also thought to explain, in part, *KLK10* silencing in ovarian and prostate cancers.[5]

Another mechanism of transcriptional control involves locus control regions, a class of *cis*-acting regulatory elements that regulate the expression of linked genes in a tissue and copy number-specific manner in a wide spectrum of mammalian gene families (169, 170), including rodent kallikrein gene families (171). Smith et al. (171) propose that a dominant locus control region controls the tissue-specific expression of all rat kallikrein genes in the salivary gland, in conjunction with gene-associated regulatory elements within promoter and enhancer regions. Given the above and the fact that all human *KLK* genes, except *KLK2* and *KLK3*, are transcribed in the same direction (from telomere to centromere) and that many are coexpressed within tissues, locus control regions may also be implicated in the coordinate regulation and expression of human kallikrein genes.

Therefore, although steroid hormones play a major role, the control of kallikrein gene transcription may involve integration of a myriad of transcription factors and pathways, including epigenetic mechanisms and locus control regions, which serve to increase regulatory diversity and provide opportunities for cell and tissue-specific responses.

Post-translational Control of Protein Function

One of the main characteristics of proteases is their ability to catalyze reactions irreversibly. As a consequence, several mechanisms have evolved to spatially and temporally regulate serine protease activity to prevent unwanted protein degradation, including: (a) zymogen activation, (b) internal cleavage, and (c) endogenous inhibitors. First, all known proteases are synthesized as zymogens or inactive precursors, which possess an inhibitory pro-peptide that sterically blocks the active site and thereby prevents substrate binding. Zymogen conversion to the active enzyme generally occurs by limited proteolysis of the pro-peptide, via diverse mechanisms, including enzymatic or non-enzymatic cofactors that trigger activation, to a simple pH change resulting in autoactivation (reviewed in ref. 172) and can occur intracellularly (within the secretory pathway) or extracellularly. All pro-hKs, expect hK4, require the activity of a trypsin-like serine protease for activation as shown in Table 2. Thus, several studies have reported activation of pro-hKs by trypsin, enterokinase, trypsin-like hKs, and via autoactivation, in vitro (to be discussed in detail in the following section).

Once activated, serine proteases may be inactivated by internal cleavage followed by degradation. Cleavage may be autolytic or mediated by another protease. This mechanism has been reported for six members of the kallikrein family. For instance, degraded forms of hK2 with a major cleavage site between residues R^{145}-S^{146} and a minor site between R^{101}-L^{102} have been isolated from seminal plasma and prostate tissues (173, 174). As well, hK3 purified from the seminal plasma (~30% of total hK3) and prostatic tissues contains internal peptide bond cleavages at one major, K^{145}-K^{146}, and two minor, R^{85}-F^{86} and K^{182}-S^{183} sites, leading to inactivation (175-177). The enzyme(s) responsible for internal cleavage of hK2 or hK3 are still unknown; however, hK2 is likely autodegraded because this enzyme possesses trypsin-like activity and the cleavage sites (P1-Arg) require a trypsin-like specificity. Furthermore, hK6 (178) and hK13 (179) are capable of autoinactivation in vitro, between residues R^{76}-E^{77} and R^{114}-S^{115}, respectively. Self-digestion has also been reported for hK7 (28) and hK14,[6] yet the cleavage sites have not been determined.

Many endogenous inhibitors are known to regulate the activity of serine proteases. Laskowski et al. (180, 181). Generally speaking, many specific inhibitors are capable of inhibiting the same serine protease, and the same inhibitor may inhibit several serine proteases (180). Many kallikreins form complexes in vivo and/or in vitro with plasma inhibitors, primarily serpins and α2-macroglobulin (α_2M) (Table 2). The interaction of serpins with serine proteases can occur via (a) the inhibitory pathway, leading to complex formation that results in the deformation and irreversible inactivation of the protease or (b) the substrate pathway, in which the serpin is cleaved by the protease and does not result in the inhibition of the protease (182, 183). hK1, 2, 3, 5, 6, and 13 form complexes and are inhibited in biological fluids such as serum, ascites fluid, seminal plasma, cerebrospinal fluid, milk of lactating women, by various serpins, including α1-antitrypsin (a.k.a. α1-protease inhibitor), α1-antichymotrypsin (ACT), protein C inhibitor (a.k.a. plasminogen activator inhibitor-3), plasminogen activator inhibitor-1, antithrombin III, and α2-antiplasmin. For instance, the major fraction (70% to 90%) of total hK3 in serum is complexed with ACT (184), whereas hK2 is

[5] Our data submitted for publication.

[6] Our unpublished data.

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complexed with ACT and protein C inhibitor in serum but at a much lower proportion (4% to 19%; refs. 185, 186) Interestingly, kallistatin is a uniquely specific serpin for hK1 (187, 188). Some kallikreins also interact with serpins via the substrate pathway, for example, hK3 with ACT (189) and hK5 and hK6 with α_2AP (ref. 178 and our unpublished data). The interaction of proteases with α_2M involves a molecular trap mechanism, which does not lead to inhibition of protease activity but prevents proteases from interacting with large substrates or inhibitors by steric hindrance (190). Human kallikreins 2, 3, 5, and 13 interact with α_2M in the serum. Furthermore, it has been proposed that inhibitory peptides derived from serine protease inhibitor Kazal-type 5 (SPINK5) may regulate hK activity due to the colocalization of *KLKs* and SPINK5 in the skin (108).

Physiologic Roles

Due to their presence in diverse tissues and cell types and by virtue of their serine protease activity, kallikreins are implicated in a wide range of normal physiologic processes, from the regulation of cell growth to tissue remodeling (Table 2). To date, several biological roles have been established for classical kallikreins, hK1, hK2, and hK3. The primary activity of hK1 involves the cleavage of low molecular weight kininogen to release lysyl-bradykinin (kallidin), which in turn binds to its receptors, bradykinin B1 and B2, in target tissues and mediates varied processes such as regulation of blood pressure, smooth muscle contraction, neutrophil chemotaxis and pain induction, vascular permeability, vascular cell growth, electrolyte balance, and inflammatory cascades (191, 192). A role for the hK1-kinin system in the establishment and maintenance of placental blood flow through vasodilation, platelet antiaggregation, cell proliferation, and trophoblast invasion during different stages of pregnancy has also been suggested (193, 194). Apart from its kininogenase activity, hK1 is implicated in other tissue and/or cell-specific functions, including processing growth factors and peptide hormones (listed in Table 2) in the pituitary, pancreas, and other tissues (191, 195-198).

Unlike hK1, both hK2 and hK3 possess relatively low kininogenase activity (199). Four in vitro studies have shown that hK2 is able to activate itself (67, 173, 200, 201). A series of contradictory studies has been published with respect to the activation of pro-hK3 by hK2 (173, 201-203). In 1997, three independent groups published that mature hK2 was able to activate pro-hK3, at a slow rate (173, 202, 203). However, in a subsequent experiment, Denmeade et al. (201) showed that hK2 was unable to cleave the fluorogenic pro-hK3 peptide substrate APLILSR-AMC, calling into question the previous findings. Once enzymatically active, hK2 and hK3 contribute to seminal clot liquefaction after ejaculation, which is integral to sperm motility, through their hydrolysis of seminal vesicle proteins, seminogelin I and II, and fibronectin (204, 205). However, hK3 cleaves these substrates at a higher efficiency and at different sites compared with hK2. As listed in Table 2, many other potential substrates, including growth factor binding proteins, peptide hormones, and components of the basement membrane/extracellular matrix (ECM), have been identified for hK2 and hK3.

Thus far, the physiologic roles of the remaining kallikreins, hK4 through hK15, have not been fully elucidated. However, putative functions have been proposed for several, based on their sites of expression and/or on the activity of orthologous proteins.

For instance, accumulating data suggest that several kallikreins may be implicated in the processing of peptide hormones in the endocrine pancreas. Immunohistochemical studies indicate that hK1, 6, 10, and 13 are all strongly expressed in the islets of Langerhans, within the specialized β, α, δ and pancreatic polypeptide cells that synthesize insulin, glucagon, somatostatic, and pancreatic polypeptide, respectively (120, 121, 127, 167, 206-208). Accordingly, these kallikreins may participate in prohormone activation, possibly in cooperation with other prohormone convertases, such as PC1 and PC2, which also colocalize with kallikreins in similar cell types of the endocrine pancreas (208). In fact, the activation of proinsulin to mature insulin by hK1 has already been documented (209).

Several reports suggest that kallikreins play a role in the normal physiology of the skin, particularly in epidermal homeostasis. Both hK5 (47) and hK7 (28) have been isolated and cloned from the stratum corneum, the outermost layer of the skin. They are proposed to function in the degradation of intercellular structures (131, 210), such as desmosomes (211-214), connecting the corneocytes, thereby decreasing cellular cohesiveness and facilitating cell shedding or desquamation during the terminal stages of epidermal turnover. However, because hK5 and hK7 preferentially cleave only a subset of desmosomal proteins (214), additional trypsin and chymotrypsin-like proteases are implicated in stratum corneum desquamation (215), including many other members of the kallikrein family (108). In addition to desquamation, hK7 may also play a role in skin pathophysiology, including pathologic keratinization (130), psoriasis (216), and in inflammatory reactions, due to its ability to activate proinflammatory cytokines, such as interleukin-1β (IL-1β; refs. 217, 218).

We have recently published a review discussing the potential roles of kallikreins in the central nervous system (219). Putative functions for hK6 and hK8 have been extrapolated from the experimentally verified actions of their rodent orthologs. Given the high amino acid sequence identity among hK6 and hK8 and their rodent orthologs (~70%), it is conceivable that the proteins exhibit similar activities. For example, the rat ortholog of hK6, called myelencephalon-specific protease (MSP), may play a role in the regulation of central nervous system demyelinating disease (220-223), including the development of multiple sclerosis lesions (221), while the mouse ortholog may function in myelination and myelin turnover (224). As well, human kallikrein 6 is implicated in the development of Alzheimer's disease partly due to its ability to cleave amyloid precursor protein, in vitro, and possibly generate β-amyloid peptides (31, 178), which are known to aggregate and form one of the major pathologic lesions characteristic of this disease. Several reports indicate that mouse hK8/neuropsin might be involved in synaptogenesis, neural development (225), regulation of long-term potentiation (LTP; refs. 226, 227) and seizures in kindled brain (228).

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Furthermore, based on the expression patterns and suggested roles of its mouse and porcine orthologs, the human hK4 protein may likely be involved in tooth development via enamel matrix protein degradation and/or processing during dental enamel formation (229-232).

Moreover, in addition to hK3 (233) and hK6 (69, 178), our preliminary data suggest that several other kallikreins, including hK5, hK13, and hK14, are able to cleave components of the ECM in vitro. Hence, kallikreins may also function in tissue remodeling, similar to matrix metalloproteases (234).

Circumstantial evidence suggests that cross-talk likely exists among members of the human kallikrein gene family and with proteases of other catalytic classes. On the basis of the colocalization of kallikrein genes to the same chromosomal locus; their coordinated regulation by steroid hormones, coexpression in tissues, and biological fluids; and the reported ability of certain kallikreins to autoactivate and potentially activate other kallikreins and proteases, it has been presumed that this family may participate in a proteolytic cascade pathway (235). The best examples of well-established enzymatic cascades involving serine proteases include the blood coagulation, fibrinolytic, and digestive cascades (236, 237). These cascades are characterized by a series of zymogen or proenzyme activations, in which the activated form of one enzyme catalyzes the activation of the following zymogen, and by the rapid amplification of the initial signal during their progression.

As illustrated in Fig. 1, all 15 kallikrein genes colocalize to 19q13.4. The colocalization of genes encoding proteins that take part in the same pathway is not uncommon in the human genome. For example, several serine proteases involved in sequential steps of the coagulation cascade are encoded by tandemly colocalized genes and some may share a common ancestor, similar to the kallikrein family (236, 238, 239). Kallikrein genes are also coordinately regulated by steroid hormones and coexpressed in similar tissues (e.g., skin, prostate, breast, pancreas) and found in biological fluids (e.g., seminal plasma, milk of lactating women) under normal conditions, as discussed above. The parallel pattern of differential kallikrein expression in malignancy, such as the concurrent up-regulation of *KLK5*, *KLK6*, *KLK7*, *KLK8*, *KLK10*, *KLK11*, and *KLK14* in ovarian cancer (240), further substantiates the possible existence of a steroid hormone-driven cascade.

As shown in Table 2, all hK pro-peptide cleavage sites contain a P1 Arg or Lys residue, with the exception of hK4, indicating that pro-hKs generally require the activity of a trypsin-like serine protease for activation. For instance, it has been shown that trypsin can convert pro-hK5, pro-hK6, pro-hK7, and pro-hK15 into their active forms, while enterokinase can activate pro-hK11 (28, 36, 47, 64, 167). Because most kallikreins possess trypsin-like specificity, many are implicated in autoactivation and the activation of other pro-hKs as well. For instance, hK2 (174, 201), hK6 (31, 178), and hK13 (179) are all capable of autoactivation and may, therefore, be involved in the initiation and maintenance of a cascade, similar to factor XI of the intrinsic coagulation pathway (241). As well, experimental evidence has shown that recombinant hK2, hK4, and hK15 can readily activate pro-PSA, in vitro (53, 173, 202, 203, 242). Brattsand and Egelrud (47) also hypothesize that hK5 may potentially activate pro-hK7 in the skin.

Kallikreins may also be implicated in additional pathways involving proteases of similar or different catalytic types. This is evident from the reported ability of hK2 and hK4 to activate the pro-form of uPA (242, 243), a serine protease that converts the serine protease, plasminogen to plasmin, which in turn degrades the ECM and activates members of the matrix metalloprotease family (244). In addition to plasmin, kallikreins may also be involved in the activation of pro-matrix metalloproteases, because matrix metalloproteases require the activity of trypsin-like serine proteases for cleavage of their pro-peptides (245, 246). Interestingly, porcine hK1 was found to activate type IV collagenase, a matrix metalloprotease family member (247). These findings implicate kallikreins in the promotion of tumor invasion and metastasis (further discussed below). Conversely, some members of the kallikrein family, namely hK3 (248), hK13 (179), and hK6[7] are able to cleave plasminogen, causing the release of biologically active angiostatin-like fragments known to inhibit angiogenesis (249).

Protease-activated receptors (PAR) comprise a small subfamily of G protein–coupled receptors, through which serine proteases mediate their hormone-like effects on cells (250). Unlike most receptors, PARs are stimulated by serine protease cleavage of an extracellular NH_2-terminal segment, generating a new NH_2-terminal sequence that acts as a tethered ligand and interacts with the second extracellular loop (251). Thus, the protease changes the conformational structure of the receptor, such that it acts as its own activator. Due to the coupling of PARs with several G protein family members, a complex network of intracellular signaling pathways may be activated, leading to changes in morphology, proliferation, survival, cell mobility, and gene transcription, shown to be important in the physiology of the vascular and nervous systems (252-254). Out of the four human PARs identified to date, PAR-1 (250), -3 (255), and -4 (256) are relatively specific for thrombin, whereas PAR-2 is not activated by thrombin and seems to have a broader range of cognate proteases, including trypsin, tryptase, and coagulation factors VIIa and Xa (257, 258). However, PAR-1 does not restrict activation by other proteases, because it may also be activated by coagulation factor Xa (259), plasmin (260), and the anticoagulant protein C (261). The absence of the unique thrombin-complementary extracellular domain from PAR-2 and PAR-4 leads to the concept of "generic PARs" and renders them as candidates for other serine proteases, including human kallikreins. Recently, a trypsin-like serine protease named P22, enzymatically similar to rat hK8, was isolated from rat brain and shown to activate PAR-2 (262). The potential involvement of human kallikreins in the activation of PARs should be explored.

Involvement in Cancer

Carcinogenesis is a complex process that involves alterations at the DNA, mRNA, and protein levels. The main goal of cancer research is to identify these alterations and determine their effects on the tumor phenotype. Accumulating reports

[7] G. Sotiropoulou, personal communication.

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indicate that the human kallikrein family is implicated in cancer. All 15 kallikrein genes are differentially expressed in cancer, primarily in hormone-related malignancies, at the mRNA and/or protein levels. For instance, numerous studies have shown that kallikreins 4, 5, 6, 7, 8, 10, 11, 13, 14, and 15 are overexpressed in ovarian carcinoma tissues, serum, and/or cell lines at the mRNA and/or protein levels (30, 34, 71, 110, 115, 116, 119, 123, 263-272). The up-regulation of kallikreins 5, 6, 7, 8, 10, 11, and 14 in ovarian cancer was further verified in silico via digital differential display and X-profiler analyses of kallikrein gene expression in normal and cancerous ovarian tissues and cell lines by Yousef et al. (240). In contrast to ovarian cancer, kallikrein genes 3, 10, 12, 13, and 14, are down-regulated in breast cancer tissues and/or cell lines at the mRNA level (29, 49, 50, 52, 125, 273-276), while the *KLK6* gene is down-regulated in metastatic breast cancer sites and up-regulated in primary breast tumors (30). In silico analyses of kallikrein mRNA expression levels in normal and cancerous breast tissues and cell lines suggests that at least four kallikrein genes, namely *KLK5*, *6*, *8*, and *10* are down-regulated in breast cancer (277), partially consistent with the previous findings. Although human kallikrein 5 and 14 mRNA levels are reduced in breast cancer, elevated serum levels of the hK5 and 14 proteins were observed in a subgroup of breast cancer patients (110, 116). This discrepancy between kallikrein 5 and 14 mRNA and serum protein levels in breast cancer has also been observed for hK3/PSA in prostate cancer. In these cases, the elevation of hK proteins in the serum may be due to angiogenesis and/or destruction of glandular architecture during carcinogenesis, thereby facilitating the outflow of hKs into the circulation. With respect to prostate cancer, *KLK2*, *KLK3*, *KLK5*, *KLK6*, *KLK10*, and *KLK13* are down-regulated compared with normal adjacent tissue (117, 122, 278-282), whereas *KLK11*, *KLK14*, and *KLK15* are overexpressed (115, 283, 284). Additionally, the expression of *KLK5*, *KLK10*, and *KLK14* is markedly reduced in cancerous versus normal testicular tissues at the mRNA level (52, 285, 286). (For a recent review describing the association of kallikreins with testicular cancer, see ref 287.)

In addition to hormone-related cancers discussed above, kallikrein expression is dysregulated in several other malignancies. A recent microarray analysis profiling the gene expression patterns in human lung adenocarcinomas indicated that *KLK11* is uniquely overexpressed in a subgroup of neuroendocrine carcinomas (288). Another microarray study has characterized differential transcription profiles in pancreatic ductal adenocarcinomas and showed that *KLK10* is one of the most highly and specifically overexpressed genes in pancreatic cancer compared with normal and benign pancreas tissues (289). Furthermore, the *KLK10* gene is down-regulated in acute lymphblastic leukemia (168).

Emerging data indicate that many alternative kallikrein transcripts are also differentially expressed in cancer and some are even cancer-specific. Dong et al. (101) have recently documented the overexpression of a *KLK5* variant with a short 5′ UTR and a *KLK7* variant with a longer 3′ UTR in ovarian cancer cell lines, compared with normal ovarian epithelial cells. A *KLK5* splice variant, denoted *KLK5* splice variant 1, is up-regulated in ovarian cancer tissues, but down-regulated in prostate cancer tissues compared with normal.[8] Two novel mRNA splice variants of the *KLK8* gene, missing either two or three coding exons, are overexpressed at relatively high levels in cancerous ovarian tissues, compared with normal ovarian tissues, in which they were not detected (267). The *KLK11* gene has two transcript variants named the brain type and prostate type (36), both of which are overexpressed in cancerous prostate versus normal tissues (290). Moreover, the work of Chang et al. (133) has revealed that the *KLK13* gene possesses at least five tissue-specific splice variants expressed exclusively the testis, in contrast to classical *KLK13* mRNA which is predominately expressed in a variety of tissues including the breast, prostate, testis, and salivary gland (49). These *KLK13* splice variants are expressed in a fraction of morphologically normal testicular tissues, but absent in the adjacent cancerous tissues (133).

The mechanisms giving rise to the differential expression of kallikrein genes in cancer have not been fully elucidated. However, epigenetic modifications, specifically the hyper-methylation of coding exon 3, are responsible for the down-regulation of *KLK10* in breast, prostate, and ovarian cancers and acute lymphoblastic leukemia (refs. 167, 168) and our data submitted for publication). This epigenetic mechanism is an important cause of gene silencing in carcinogenesis (291). Also, considering that steroid hormones are implicated in the etiology of hormone-related malignancies, such as ovarian, breast, prostate, and testicular cancers (138), and are also known to regulate kallikrein gene expression, it may be possible that kallikreins are part of a steroid hormone–driven (cascade) pathway that is activated during the promotion and progression of cancer.

With respect to their involvement in the pathogenesis of cancer, kallikreins seem to have a dual role, because they can either promote or inhibit carcinogenesis. For one, many kallikreins are directly and/or indirectly involved in the degradation of ECM proteins, which facilitates tumor invasion and metastasis. As listed in Table 2, hK2, 3, 5, 6, 13, and 14 can directly catalyze the hydrolysis of several ECM proteins (refs. 69, 178, 204, 205, 233 and our unpublished data). hK2 and hK4 are indirectly involved via activation of uPA, leading to plasminogen activation, ultimately resulting in ECM degradation (242, 243). Furthermore, a synthetic hK1 inhibitor was recently found to suppress the invasiveness in human breast cancer cell lines by 33% in matrigel invasion assays (292), suggesting that it has a role in facilitating tumor cell migration, via ECM cleavage. These findings are further supported by the numerous reports indicating that kallikrein overexpression is associated with poor prognosis in cancer patients (Tables 4-6). As well, hK3 can also cleave insulin-like growth factor binding protein 3 (IGFBF-3), thus, liberating insulin-like growth factor, which is a mitogen for prostatic stromal and epithelial cells (293) and activate transforming growth factor β, thereby stimulating cell detachment and facilitating tumor spread (294). hK1 is present in colon, breast, lung, stomach, pituitary, uterine, and esophageal cancer cells and may be

[8] Our data submitted for publication.

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involved in malignant transformation by stimulating proliferation of tumor cells and increasing vascular permeability (148, 198, 295-302). hK1, via kinin action, also enhances vascularity, mitogenicity, metastasis, and regulates angiogenesis (303-305).

Kallikreins may also inhibit carcinogenesis. *KLK10* is thought to be a tumor suppressor gene, by virtue of its down-regulation in several cancers (29, 125, 168, 276) and because transfection of this gene into the tumorigenic breast cancer cell line MDA-MB-231 reduced its anchorage-independent growth and the tumor formation of nude mice inoculated with this *KLK10*-transfected cell line was significantly reduced. hK3 may also act as a tumor suppressor, an inducer of apoptosis (306), and as a negative regulator of breast cancer cell growth (307). hK3, 6, and 13 are also implicated in the inhibition of angiogenesis, via their release of angiostatin-like fragments from plasminogen (refs. 179, 248, 308 and G. Sotiropoulou, personal communication). These studies may help to explain, in part, why certain kallikreins are markers of favorable prognosis for cancer patients (Tables 4-6).

Clinical Applications

Cancer biomarkers are fundamental tools that aid in evaluating cancer risk, screening, diagnosis, clinical staging, estimating tumor volume, monitoring, assessing prognosis, evaluating success of treatment, detecting disease recurrence, and predicting a likely response to therapy to improve patient management and outcomes (309). Among all biomarkers to date, hK3/PSA has had the greatest impact in clinical practice, for the screening, diagnosis, staging, and monitoring of prostate cancer (310). Most other biomarkers often lack the desired sensitivity and specificity and, thus, the search for more informative markers continues.

The tissue kallikrein family has proved to be a rich source of cancer biomarkers (311-313). In addition to hK3, many other kallikreins exhibit altered mRNA and/or protein expression levels within the tissues and/or serum of cancer patients and represent prospective biomarkers for early detection, prognosis, or monitoring of certain hormone-dependent malignancies, as summarized in Tables 4-6. For instance, serum hK2 may function as an alternate or complementary biomarker to hK3 for prostatic diseases (314, 315). Importantly, this kallikrein may aid in the differential diagnosis between prostate cancer and benign prostatic hyperplasia (316) as well as the identification of organ-confined versus non-organ–confined disease (317). With the recent developments of highly sensitive and specific immunoassays for hK5, hK6, hK8, hK10, hK11, and hK14 proteins, elevated levels of these kallikreins were observed in the tissues and/or serum of a proportion of ovarian cancer patients (110, 113, 115, 116, 266, 270, 271, 318). Pre-surgical serum hK6 and hK10 levels increase the diagnostic sensitivity of CA125 in patients with early stage (I/II) ovarian cancer and are associated with poor patient prognosis. Thus, serum hK6 and hK10 may complement CA125 for early detection of ovarian cancer. Serum hK5 and hK14 levels are also increased in ~40% of women with breast cancer, whereas serum hK11 is elevated in 60% of men with prostate cancer. The latest study by Nakamura et al. (319) also shows that serum hK11 levels and the hK11/total PSA ratio are both significantly lower in patients with prostate cancer than in BPH patients, suggesting

Table 4. Human Kallikreins as Ovarian Cancer Biomarkers (Messenger RNA and/or Protein Level)

Kallikrein Gene (*KLK*)/ Protein (hK)	Samples Used	Clinical Applications	References
KLK4	mRNA from normal and cancerous ovarian tissues	Unfavorable prognostic marker	(119, 263)
KLK5	mRNA from normal and cancerous ovarian tissues	Unfavorable prognostic marker	(264)
KLK5/hK5	mRNA and cytosolic extracts from normal and cancerous ovarian tissues	Unfavorable prognostic marker	(101)
hK5	ovarian cancer cytosols	Unfavorable prognostic marker	our data submitted for publication
	serum and tissue	Marker of diagnosis	(110)
KLK6/hK6	mRNA and extracts from normal, benign, and cancerous ovarian tissues	Unfavorable prognostic marker	(265)
hK6	ovarian cancer cytosols	Unfavorable prognostic marker	(323)
	serum	Marker of diagnosis, prognosis, and monitoring	(266, 318)
KLK7	mRNA from cancerous ovarian tissue	Unfavorable prognostic marker	(324)
KLK7/hK7	mRNA and extracts from normal and cancerous ovarian tissues	Unfavorable prognostic marker	(101, 123)
KLK8	mRNA from ovarian cancer tissues	Favorable prognostic marker	(267)
hK8	serum and tissue	Marker of diagnosis, prognosis, and monitoring	(268)
KLK9	mRNA from ovarian cancer tissues	Favorable prognostic marker	(124)
hK10	serum and tissue	Marker of diagnosis, prognosis, and monitoring	(270, 271)
	normal, benign, and cancerous ovarian cytosols	Unfavorable prognostic marker	(269)
hK11	ovarian cancer cytosols	Favorable prognostic marker	(325)
	serum	Marker of diagnosis	(115)
hK13	ovarian cancer cytosols	Favorable prognostic marker	(326)
KLK14	mRNA from normal, benign, and cancerous ovarian tissues	Favorable prognostic marker	(147)
hK14	serum and tissue	Marker of diagnosis	(116)
KLK15	mRNA from benign and cancerous ovarian tissues	Unfavorable prognostic marker	(272)

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Table 5. Human Kallikreins as Breast Cancer Biomarkers (Messenger RNA and/or Protein Level)

Kallikrein Gene (*KLK*)/Protein (hK)	Samples Used	Clinical Applications	References
hK3	serum and tissue	Marker of diagnosis and prognosis	reviewed in ref. 402
KLK5	mRNA from breast cancer tissues	Unfavorable prognostic marker	(158)
hK5	serum	Diagnostic marker	(110)
KLK7	mRNA from breast cancer tissues	Unfavorable prognostic marker	(328)
KLK9	mRNA from breast cancer tissues	Favorable prognostic marker	(330)
hK10	breast cancer cytosols	Predictive value	(333)
KLK13	mRNA from breast cancer tissues	Favorable prognostic marker	(331)
KLK14	mRNA from breast cancer tissues	Unfavorable prognostic marker	(329)
hK14	serum and tissue	Diagnostic marker	(116)
KLK15	mRNA from breast cancer tissues	Favorable prognostic marker	(158)

that serum hK11/total PSA may aid in differential diagnosis. Using the hK11/total PSA ratio at 90% sensitivity, it would be possible to avoid ~50% unnecessary prostatic biopsies not evaded by the %free-PSA test (%free PSA <20). Furthermore, complexed forms of hK2 and hK3 with various plasma inhibitors (320), degraded forms of hK2 and hK3 (321), and the altered glycosylation patterns observed between hK3 produced by normal and cancerous prostate tissues (322), may also have clinical utility in prostate cancer diagnostics and prognostics.

In addition to their clinical value as serologic biomarkers, kallikrein mRNA and protein levels within cancerous tissues are often associated with patient prognosis. As listed in Table 4, *KLK4*, *KLK5*/hK5, *KLK6*/hK6, *KLK7*, hK10, and *KLK15* are markers of poor prognosis in ovarian cancer (263-265, 269, 271, 272, 323, 324). That is, higher kallikrein mRNA and/or protein levels were found to correlate with more aggressive forms of this disease and a decreased disease-free and overall survival. Conversely, the remaining subset of kallikreins, namely *KLK8*, *KLK9*, hK11, hK13, and *KLK14*, are markers of favorable prognosis (124, 147, 267, 325, 326). Higher levels of their mRNA or protein levels predominate in earlier stage disease and are associated with increased disease-free and overall survival. The expression of these kallikreins in ovarian cancer may also be clinically useful in determining the prognosis in subgroups of patients. For instance, a subgroup of kallikreins (kallikreins 4, 6, and 10) are highly expressed in serous epithelial ovarian tumors, whereas higher expression of another group (kallikreins 5, 11, and 13) is more frequently found in non-serous tumors. These data suggest that certain kallikreins may be used as determinants of prognosis in the subgroups of ovarian cancer patients stratified by histotype as well.

With respect to breast cancer (Table 5), the mRNA expression levels of *KLK5*, *KLK7*, and *KLK14* in breast tumors are indicative of a poor patient prognosis (327-329), while higher levels of *KLK9*, *KLK13*, and *KLK15* mRNA and the hK3 protein forecast a favorable disease outcome (158, 275, 330, 331). Furthermore, high levels of hK3 and hK10 proteins in breast carcinomas are significantly related to a poor response to tamoxifen therapy (332, 333).

Several kallikreins also have prognostic/predictive value in prostate carcinoma (Table 6). For example, lower tissue hK3 concentration is associated with more aggressive forms of this cancer, such that tumors expressing high levels are associated with a favorable prognosis (279, 334). Higher *KLK5* and *KLK11* mRNA levels also indicate a favorable prognosis (281, 290). Moreover, hypermethylation of the *KLK10* gene in coding exon 3 is an independent prognostic marker of decreased disease-free survival in children and adults and in the separate analysis of adults with acute lymphoblastic leukemia (168).

The discovery of cancer-specific mRNA transcript variants that occur exclusively or with higher frequency in cancer cells and which are detectable by biopsy or in body fluids, may serve as useful diagnostic biomarkers. The most well-characterized cancer-specific splice variant biomarkers include those of the CD44 and Wilm's tumor (WT1) genes (335, 336). As previously discussed, preliminary studies indicate that variant kallikrein transcripts are differentially expressed and/or expressed specifically in cancer and may, therefore, constitute a new generation of cancer biomarkers within the kallikrein family.

For instance, Slawin et al. (337) have recently developed a preoperative *KLK2* splice variant–specific reverse transcription-PCR that is useful for detecting prostate cancer metastasis and helps predict pathologic lymph node positivity in men with clinically localized prostate cancer. Tanaka et al. (105) have reported the existence of an alternatively spliced form of the *KLK3* gene that is expressed in 13 of 18 (72.2%) noncancerous and 4 of 5 (80.0%) cancerous prostate tissues, but in only 3 of

Table 6. Human Kallikreins as Prostate Cancer Biomarkers (Messenger RNA and/or Protein Level)

Kallikrein Gene (*KLK*)/ Protein (hK)	Samples Used	Clinical Applications	References
hK2	serum and tissue	Marker of diagnosis, prognosis, and monitoring	(315, 321, 403)
hK3	serum and tissue	Marker of diagnosis, prognosis, and monitoring	(321)
KLK5	mRNA from matched normal and prostate cancer tissues	Favorable prognostic marker	(281)
KLK11	mRNA from matched normal and prostate cancer tissues	Favorable prognostic marker	(290)
hK11	serum	Diagnostic marker	(319)

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12 (25.0%) blood samples from prostate cancer patients. The difference in *KLK3* variant expression levels between noncancerous prostate tissues versus blood samples from cancer patients was statistically significant ($P = 0.011$). David et al. (132) have reported the identification of two splice variants of the *KLK2* and *KLK3* genes that result from inclusion of intronic sequences adjacent to the first exon, denoted K-LM and PSA-LM, respectively. With the exception of the signal peptide, K-LM and PSA-LM transcripts encode protein isoforms that are entirely different than the classical hK2 and hK3 proteins. As such, polyclonal antibodies were generated against synthetic peptides derived from amino acid sequences unique to each variant protein. Immunohistochemistry of prostate sections using these polyclonal antibodies indicated that the K-LM and PSA-LM proteins are detected only in the secreting cells of the tubule lumen and Western blot analysis indicated that the K-LM protein is present in seminal plasma, similar to the classical forms of hK2 and hK3. Furthermore, a recent study indicates that *KLK3* may actually produce at least 15 transcripts, which can encode eight putative protein isoforms (97). Reverse transcription-PCR analysis indicates that at least five splicing isoforms are expressed in normal, benign prostatic hyperplastic, and cancerous tissues. Collectively, these *KLK2* and *KLK3* variants may supplement hK3/PSA diagnostics. Using quantitative reverse transcription-PCR, Nakamura et al. (290) compared the expression of the prostate and brain-type *KLK11* isoforms in matched normal and cancerous prostatic tissues. Both variants were overexpressed in cancerous prostate versus normal tissues and lower expression of prostate-type *KLK11* was associated with higher tumor stage, grade and Gleason score. No such association was seen with the brain-type isoform. These data suggest that *KLK11* splice variants may have clinical value as biomarkers for prostate cancer diagnosis and prognosis. Variant transcripts of *KLK5*, *8*, and *13* are also differentially expressed in cancer, as discussed in a previous section. The biological and clinical significance of these variant kallikrein transcripts/proteins remains to be elucidated.

Recently, it has become possible to combine the diagnostic, prognostic, and predictive value of multiple biomarkers into models, which have the ability to discriminate better than single biomarkers alone (338-342). For example, the use of logistic regression, decision tress, discriminant analysis, and artificial neural networks can outperform single biomarker analysis in diagnostic, prognostic, and predictive applications. Therefore, the combination of a subset of classical and/or variant kallikreins into a multiparametric panel may provide superior diagnostic/prognostic information than that of the single analytes alone. Further studies are warranted to evaluate this hypothesis.

Single-nucleotide polymorphism (SNP) within candidate genes can affect coding sequences, transcriptional regulation, and splicing and may confer increased susceptibility or resistance to complex diseases, such as cancer. As such, SNPs are considered potential markers of cancer risk and progression and can help to define resistance to therapeutic regimens. Several SNPs have been reported in the human kallikrein locus, within *KLK1* (343), *KLK2* (344), and *KLK10* (345) genes and the *KLK3* (155, 346, 347) promoter region. The *KLK2* SNP (C→T) in exon 5 changes the amino acid from Arg^{226} to Trp^{266}, leading to an active (C allele; Arg^{226}) and inactive (T allele; Trp^{266}) form of hK2 (344). A recent study has found a strong positive relationship between this polymorphism with serum hK2 levels and prostate cancer risk, that is, the T allele is associated with lower hK2 levels, but a higher risk of cancer (348). Regarding *KLK3*, three SNPs are in the proximal promoter at positions −158, −205, and −252, which may be implicated in breast and/or prostate cancer susceptibility. For example, concerning the SNP at position −158 (G→A), individuals homozygous for the G allele showed higher hK3 tumor concentrations and an increased overall survival than those homozygous for the A allele (349). Depending on the study, either the G or A allele of SNP −158 was also shown to be associated with the risk of advanced prostate cancer or an earlier onset of prostate cancer in Caucasian men (350-352), an association not found in a study involving Japanese men (353). The polymorphisms at positions −205 and −252 may be associated with mRNA expression levels of *KLK3* (354), whereas the −252 SNP was not linked to prostate cancer risk and progression in two separate Japanese studies (353, 355). Lastly, Bharaj et al. (345) have identified a few SNPs within exons 3 and 4 and intron 5 of the *KLK10* gene. The most significant SNP is in codon 50 within exon 3 (C→T) and changes the amino acid in this position from Ala to Ser. The prevalence of the T allele was significantly higher in prostate cancer patients in comparison to control subjects, and may, therefore, be associated with prostate cancer risk (345).

Kallikreins may also constitute potential drug targets, therapeutic agents, and candidates for passive or active immunotherapy, once their biological pathways are delineated. For instance, the identification of CD4 positive T cells specific for naturally processed *KLK4*-derived epitopes within the T-cell repertoire of normal males support the use of *KLK4* as a target for whole gene-, protein-, or peptide-based vaccine strategies against prostate cancer (356). This kallikrein may also represent a target for immunotherapy because anti-hK4 antibodies were only present in the serum of males with prostate cancer (357).

Conclusions and Future Directions

With the discovery of the complete hK family, comprising a total of 15 serine protease genes on 19q13.4, the genomic era of kallikrein research is nearing its end. On the basis of tissue expression patterns and putative substrates, tissue kallikreins are implicated in diverse physiologic processes, from the regulation of cell growth to tissue remodeling, where they may act individually or in cascade pathway(s). Countless reports have also indicated an association between dysregulated kallikrein expression and cancer and the carcinogenic process and the potential use of kallikreins as diagnostic/prognostic biomarkers for cancer. However, many questions remain unanswered with respect to the exact role of many tissue kallikreins in normal and pathophysiology. The future must encompass the identification of physiologic substrates, delineation of the functional intersections between kallikreins and other proteolytic systems including those involved in cell signaling, a better understanding of modes of regulation, and unveiling the relevance of the complete kallikrein transcriptome and proteome including variant mRNA transcripts and proteins.

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With respect to cancer biomarker and drug discovery, the tissue kallikrein family is a gold mine waiting to be fully unearthed. Therefore, another important goal for the future will involve further defining the clinical utility of kallikreins as biomarkers for cancer, as single analytes or in combination with several suitable molecules in a multiparametric model. Thus, the post-genomic era poses a new set of challenges for research in this subgroup of the human degradome.

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