

# **HUMAN TISSUE KALLIKREINS: FROM GENE STRUCTURE TO FUNCTION AND CLINICAL APPLICATIONS**

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## 1. Introduction

Kallikreins are serine proteases with diverse physiological functions. Until recently, it was thought that the human kallikrein gene family included only three members, but recent studies have led to the complete characterization of the human kallikrein gene locus and identification of all 15 members of this family. Kallikreins are expressed in many organs, most prominently in endocrine-related tissues such as the prostate, breast, ovary, uterus, vagina, and testis. Many kallikreins are regulated by steroid hormones in cancer cell lines, and several lines of investigation have supported a link between kallikreins and cancer.

Prostate-specific antigen (PSA, hK3) and, more recently, human glandular kallikrein 2 (hK2) are used as tumor markers for prostate cancer. Several

other kallikreins, including hK5, hK6, hK8, hK10, hK11, and hK14, are emerging as new serum biomarkers for ovarian cancer diagnosis and prognosis. Some other kallikreins are differentially expressed at both the mRNA and protein levels in various endocrine-related malignancies. The coexpression of many kallikreins in several cancer types and other information points to the possibility of their involvement in a cascade-like pathway that may be associated with cancer pathogenesis or progression. Finally, in addition to their diagnostic/prognostic utilities, kallikreins may be attractive novel therapeutic targets.

## **2. Discovery of the Human Tissue Kallikrein Gene Locus: A Short Historical Perspective**

Until approximately 1998, it was thought that the human kallikrein gene locus included three genes: pancreatic/renal kallikrein (*KLK1*), glandular kallikrein (*KLK2*), and PSA (*KLK3*). Several early estimates of the size of the human kallikrein gene family were contradictory. Southern blot analysis indicated that the size of this family varied from just three to four genes [1] to as many as 19 genes [2]. Starting around 1996, several independent groups reported the cloning of new serine proteases that colocalized at the chromosomal region 19q13.4 and shared a high degree of homology with the known kallikreins (also called the “classical” kallikreins in this review). Extensive work from our laboratory and from other groups analyzing genomic sequences in the vicinity of chromosome 19q13.4, available through the Human Genome Project, has led to the cloning of all 15 members of the human kallikrein gene family. Table 1 summarizes information from genomic and protein databases for all tissue kallikreins. Later, we present the detailed description of the locus and contrast these data with information derived from the independent analysis of the DNA sequence of chromosome 19 [3].

## **3. Kallikreins in Rodents and Other Species**

Kallikreins are found in both primates and nonprimates. Kallikrein genes and proteins have been identified in six different mammalian orders: Primates, Rodentia, Carnivora, Proboscidea, Perissodactyla, and Artiodactyla [4]. The number of kallikreins varies among species, and kallikreins in rodents and other animal species have been extensively described in a number of excellent reviews [2, 5–8]. In this section, we provide only a quick overview and some recent updates about kallikrein families in different species, with special emphasis on their structural and localization relationships with the

TABLE 1  
OFFICIAL AND OTHER GENE AND PROTEIN NAMES FOR MEMBERS OF THE HUMAN KALLIKREIN GENE FAMILY<sup>a</sup>

Official gene name	Other names/symbols	Genbank accession number	Unigene cluster	Merops ID	SwissProt ID
<i>KLK1</i>	Pancreatic/renal kallikrein, hPRK	M25629 M33105	Hs.123107	S01.160	Q07276
<i>KLK2</i>	Human glandular kallikrein 1, hGK-1	M18157	Hs.181350	S01.161	P20151
<i>KLK3</i>	Prostate-specific antigen, PSA, APS	X14810 M24543 M27274	Hs.171995	S01.162	P07288
<i>KLK4</i>	Protease, KLK-L1, EMSP1, PRSS17, ARM1	AF113141 AF135023 AF148532	Hs.218366	S01.251	Q9Y5K2
<i>KLK5</i>	KLK-L2, HSCTE	AF135028 AF168768	Hs.50915	S01.017	Q9Y337
<i>KLK6</i>	Zyme, Protease M, Neurosin, PRSS9	AF013988 AF149289 U62801	Hs.79361	S01.236	Q92876
<i>KLK7</i>	HSCCE, PRSS6	D78203 L33404	Hs.151254	S01.300	P49862
<i>KLK8</i>	Neuropsin; Ovasin; TADG-14, PRSS19, HNP	AF166330 AB009849 AF095743	Hs.104570	S01.244	O60259
<i>KLK9</i>	KLK-L3 protein	AB010780			
<i>KLK10</i>	NES1, PSSSL1	AF055982 AF135026 AF055481 NM_002776	Hs.448942 Hs.69423	S01.307 S01.246	Q9UKQ9 O43240
<i>KLK11</i>	TLSP/Hippostasin, PRSS20	AB012917	Hs.57771	S01.257	Q9UBX7
<i>KLK12</i>	KLK-L5 protein	AF135025	Hs.159679	S01.020	Q9UKR0
<i>KLK13</i>	KLK-L4 protein	AF135024	Hs.165296	S01.306	Q9UKR3
<i>KLK14</i>	KLK-L6 protein	AF161221	Hs.283925	S01.029	Q9P0G3
<i>KLK15</i>	prostinogen, HSRNASPH	AF303046	Hs.250770	S01.081	Q9H2R5

<sup>a</sup>According to the Human Gene Nomenclature Committee (<http://www.gene.ucl.ac.uk/nomenclature>).

human kallikreins. More detailed discussion can be found in our recent review [9].

### 3.1. THE MOUSE KALLIKREIN GENE FAMILY

In the mouse, kallikreins are represented by a large multigene family, initially thought to include 25 genes. Among those, at least 14 genes were presumed to be encoding for serine proteases [5]. Recent data from the Mouse Genome Project (<http://www.ncbi.nlm.nih.gov/genome/guide/mouse/>) indicate the existence of 36 mouse kallikrein genes (including annotated genes). Olsson and Lundwall published the organization of the kallikrein gene family in the mouse through sequence analysis of different databases [10]. Mouse tissue kallikrein genes reside in a locus that spans approximately 310 kb at or near the Tam-1 locus on mouse chromosome 7. Data from the human/mouse homology maps (<http://www.ncbi.nlm.nih.gov/projects/Homology/>) show that this region is highly syntenic to the human chromosome 19q13.4, which harbors the human kallikrein genes (up to 75% sequence similarity). Sequence analysis indicated the presence of possible mouse orthologues for the human kallikreins 1 and 4 through 15, but not for the classical kallikreins *KLK2* and *KLK3*. Comparing the human and mouse kallikrein loci, however, indicated that although the distance between the human *KLK1* and *KLK15* genes is only 1.5 kb, the same area in the mouse genome is 290 kb in length and harbors the rest of the mouse kallikreins [10]. All mouse kallikrein genes are transcribed in the same direction and share a high degree of structural homology at both the mRNA and protein levels (70%–90%). They also share the same genomic organization, being formed of five coding exons and four introns, with completely conserved exon–intron splice sites. A TATA box variant “TTAAA” and consensus polyadenylation signal sequences were found in all mouse kallikreins [11–13]. All mouse kallikreins code for pre-pro-kallikreins that are 261 amino acids in length, with an 18–amino acid signal peptide followed by a profragment of six amino acids. Similar to the human family, only one mouse kallikrein (*mGK6*) has a true kininogenase activity [11]. Other mouse kallikrein proteins have different types of activities. *mGK3* and *mGK4* are nerve growth factor–binding and nerve growth factor–processing enzymes; *mGK9*, *mGK13*, and *mGK22* are epidermal growth factor–binding proteins; *mGK22* is a nerve growth factor–inactivating enzyme; *mGK16* is  $\gamma$ -renin and *mGK26* is prorenin-converting enzyme-2 [14, 15]. With the expansion of the mouse kallikrein gene family and the identification of new mouse orthologues of the human kallikreins, a revision of the mouse, human, and other species’ kallikrein nomenclature, based on map sequence and evolutionary analyses, should be considered in the future.

### 3.2. THE RAT KALLIKREIN GENE FAMILY

Another large family of about 13 kallikreins was originally identified in the rat, of which at least 10 are transcriptionally active [16]. More recent data from the Rat Genome Database (<http://ratmap.gen.gu.se/>) show the possibility of the existence of additional kallikreins in the rat genome (see following). Rat kallikreins are clustered in the same chromosomal region and share a high degree of structural similarity. They also have the same conserved structure of five coding exons and four introns, with most of the similarity in the exonic, rather than intronic, regions [17]. Ten of the 13 rat kallikreins code for potentially active serine proteases that are 261 amino acids in length; the rest are assumed to be pseudogenes. As is the situation in the human and the mouse, only one rat kallikrein (*rKLK1*) meets the functional definition of a kallikrein [18]. *rKLK2* codes for tonin, which converts angiotensinogen to angiotensin II [19], and *rKLK10* codes for a kininogenase, which cleaves T-kininogen to release T-kinin [19]. Rat kallikrein mRNA levels were found to be responsive to hormonal manipulation, and castration of male animals resulted in a decrease of mRNA, which could be restored by testosterone [20]. Pituitary *rKLK1* has been found to be upregulated by estrogen [8]. Interestingly, rodent kallikreins are mainly expressed in the salivary gland, with very few of them having a wider tissue expression pattern [8].

Recently, Olsson *et al.* reported a more precise genomic organization of the rat kallikrein locus [21]. The rat *KLK* locus spans approximately 580 kb on chromosome 1q21, contains 22 genes and 19 pseudogenes, and is devoid of *KLK2* and *KLK3* orthologues. This locus contains nine duplications of an approximately 30-kb region harboring the *KLK1*, *KLK15*, and pseudogene  $\Psi$ *KLK2*, and *KLK4*, resulting in nine paralogues of each gene. However, only the *KLK1* paralogues seem to be functional. For more details, see Olsson *et al.* (2004) [21].

### 3.3. THE MASTOMYS KALLIKREIN GENE FAMILY

Mastomys is an African rodent that is intermediate in size and that has physical characteristics between the mouse and rat. It has been studied because of the presence of an androgen-responsive prostate in the female. Fahnestock reported the cloning of cDNAs from Mastomys. Two of these cDNAs were expressed in the kidney as well as the submandibular gland, and one is hypothesized to code for a true tissue kallikrein [22]. A third kallikrein was found only in the submandibular gland. DNA sequence analysis and hybridization studies demonstrate that Mastomys represents an interesting hybrid between mouse and rat [22].

### 3.4. THE MONKEY AND CHIMPANZEE KALLIKREIN GENE FAMILIES

A cynomolgus monkey tissue kallikrein gene has been characterized from a monkey renal cDNA library and has been shown to be 90% homologous to its human counterpart at the nucleotide level [23]. The *cmKLLK1* encodes a tissue kallikrein of 257 amino acids, which is 93% homologous to the human kallikrein protein. The key residues important for kininogenase activity are entirely conserved. A rhesus monkey prostatic *KLK3* cDNA encoding the simian counterpart of PSA (*KLK3*) has also been cloned [24]. It consists of 1515 nucleotides, encoding a preproenzyme of 261 amino acids, with a long 3' untranslated region. The deduced amino acid sequence is 89% homologous to human hK3 and 71% to human hK2. Tyr<sup>93</sup>, a residue important for the kininogenase activity of the human kallikrein 1, is replaced by a serine, indicating that *rmKLLK3* will lack kininogenase activity, as does its human counterpart.

Recent data from the Chimpanzee Genome Project have revealed that the chimpanzee *KLK* locus is strikingly similar to the human locus, spans about 350 kb of genomic sequence on chromosome 20, and contains orthologues to all human kallikrein genes, with an overall >99% sequence similarity at the DNA and amino acid levels (our unpublished data).

### 3.5. THE DOG KALLIKREIN GENE FAMILY

Only two kallikreins have been identified in the dog: *dKLLK1*, encoding a true tissue kallikrein based on functional definition, and *dKLLK2*, encoding a canine arginine esterase [25]. *dKLLK1* encodes a polypeptide of 261 amino acids with a typical 24-residue pre-pro-peptide, a conserved catalytic triad of serine proteases, and a tissue kallikrein substrate-binding pocket. A prostatic cDNA and the gene encoding for canine arginine esterase (*dKLLK2*) have both been cloned. As for all other mammalian kallikrein genes, *dKLLK2* consists of five exons and four introns, with fully conserved exon/intron boundaries, and an "AGTAAA" polyadenylation signal identical to that of human hK1. The *dKLLK2* gene product shows a wide pattern of tissue expression and has less overall conservation (~50%) with kallikreins of other species.

### 3.6. KALLIKREINS IN OTHER SPECIES

No more than three tissue kallikreins were identified up to now in the guinea pig [26]. Two kallikreins were cloned in the horse—a renal kallikrein [27] and a horse prostate kallikrein [28], which is a homologue of human PSA. Southern blot analysis data detected *KLK2*- and *KLK3*-positive bands in several nonhuman primate species including macaque, orangutan,

chimpanzee, and gorilla, but not in cows and rabbits [29]. Kallikreins were also isolated from the pancreas, colon, and submandibular gland of the cat [30]. Nonprimates do not contain any prostate-localized proteins homologous to PSA [31].

Completion of many other future genome projects will facilitate accurate comparisons of the *KLK* families in many species.

## **4. Characterization and Sequence Analysis of the Human Tissue Kallikrein Gene Locus**

### **4.1. LOCUS OVERVIEW**

The first comprehensive attempt to characterize the human kallikrein locus was reported by Riegman *et al.* [32], who proposed that the locus is formed of only three genes, *KLK1*, *KLK2*, and *KLK3*. These three genes were found to be clustered in a 60-kb region on chromosome 19q13.4. Their alignment in the genome is centromere-*KLK1-KLK3-KLK2*-telomere. *KLK2* and *KLK3* are transcribed in the direction from centromere to telomere. *KLK1* is transcribed in the opposite direction [32, 33].

Recently, with the discovery of all human kallikrein genes, we were able to fully characterize the human kallikrein gene locus with high accuracy and to precisely localize each of the 15 members of the human tissue kallikrein gene family, as well as to determine the distances between them and their directions of transcription [34]. The human tissue kallikrein gene locus spans a region of 261,558 bp on chromosome 19q13.4 and is formed of 15 kallikrein genes with no intervening nonkallikreins. More recently, a potential kallikrein-processed pseudogene has been cloned [34a], and the possibility exists for the presence of at least another two pseudogenes [35]. The kallikrein gene family is flanked centromerically by the testicular acid phosphatase gene [36] and telomerically by the *CAG* (cancer-associated gene) [37] and the Siglec family of genes [38]. The latest analysis of the genomic region confirmed and extended our previous findings, as shown in Fig. 1 and Table 2. Minor differences are a result of discrepancies in noncoding regions.

The kallikrein genes are clustered together, and the distances between two adjacent genes range from 1.5 (*KLK1* and *KLK15*) to 32.5 kb (*KLK4* and *KLK5*). Detailed information about the locus is presented in Fig. 1 and Table 2. The locus has been extensively analyzed for the presence of other kallikreins [34, 35, 39]. It is thus unlikely that new members will be identified inside the locus or on either end, with the possible exception of more pseudogenes.



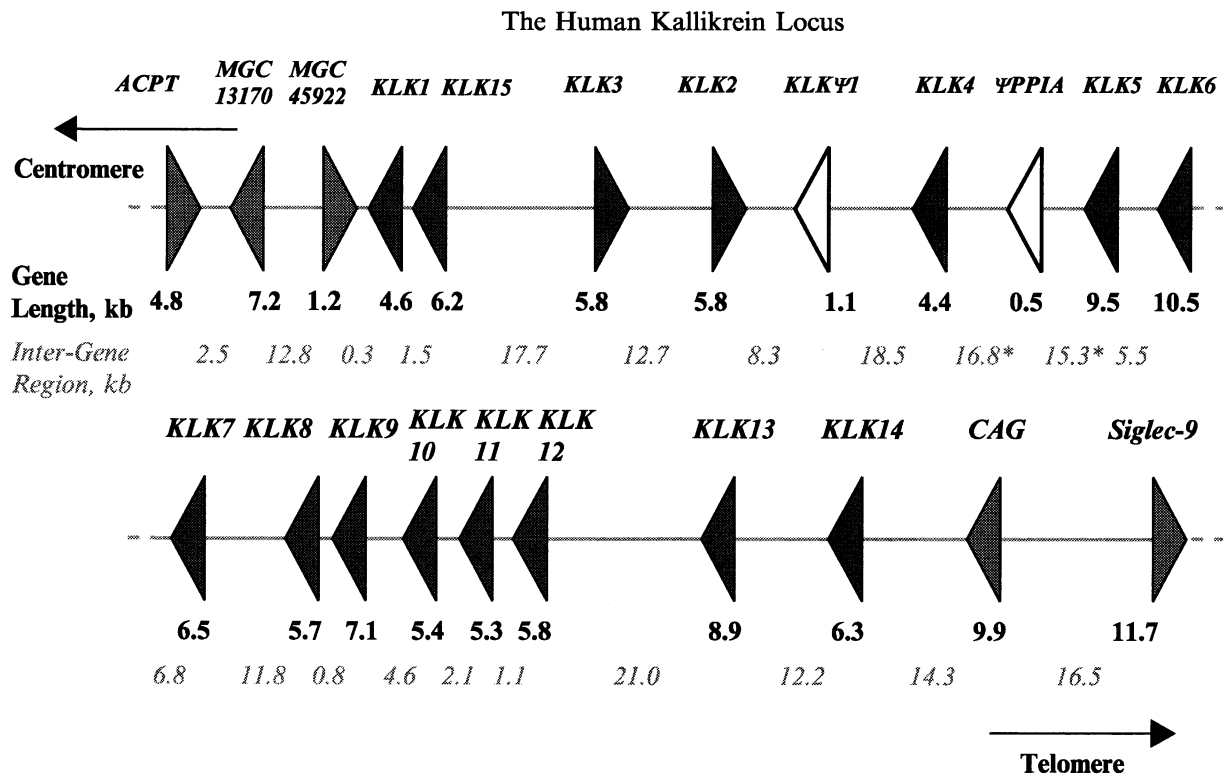


FIG. 1. Schematic representation of the human kallikrein gene locus on chromosome 19q13.4. Genes are represented by arrowheads indicating the direction of transcription. Kallikrein genes are shown in blue, and nonkallikrein genes and pseudogenes are presented by grey and white arrows, respectively. Official gene symbol is shown above each gene, and the approximate gene length is shown below each gene in kilobytes. The approximate intergenic regions are shown in red in Kb. CAG, cancer associated gene (GenBank accession AY279382); ACPT, testicular acid phosphatase (GenBank accession AF321918). The position of the PPIA pseudogene is provisional and the length of the intergenic regions (shown with asterisks) may change in the future. MGC, mammalian gene collection; these two genes have not as yet been characterized. (See Color Insert.)

#### 4.2. REPEAT ELEMENTS AND PLEOMORPHISM

The kallikrein locus was also analyzed for the presence of repeat elements [40]. The entire sequence has 49.59% GC content, which is comparable to other genomic regions. Approximately 52% of the region was found to contain various repetitive elements (on either strand). Short interspersed nuclear elements, such as ALU and MIR repeats, are the most abundant repetitive elements (22.53%), followed by the long interspersed nuclear elements, which represent 13.1% of the repetitive elements. Other repeat elements, including Tigger2, MER8, and MSR1, were also identified in *KLK4* introns [41].

The human kallikrein locus contains a unique minisatellite element that is restricted to chromosomal band 19q13, and ten clusters of this minisatellite are distributed along the kallikrein locus. These clusters are mainly located

TABLE 2  
 COORDINATES OF ALL GENES AND PSEUDOGENES CONTAINED WITHIN THE HUMAN  
 KALLIKREIN LOCUS NUMBERS ARE GIVEN ACCORDING TO GENBANK CONTIG NT\_011109

Name	Strand	Start	End	Length	Number of exons	Intergene region
ACPT	+	23561862	23566671	4810	11	2479
MGC13170	-	23576164	23569151	7014	2	12962
MGC 45922	+	23589127	23590324	1198	2	269
KLK1	-	23595233	23590594	4640	5	1501
KLK15	-	23608659	23596735	11925	5	17701
KLK3	+	23626361	23632210	5850	6	12668
KLK2	+	23644879	23650051	5173	5	8299
KLK $\Psi$ 1	-	23659265	23658351	915	1	18532
KLK4	-	23682184	23677798	4387	6	16794
$\Psi$ PPIA <sup>a</sup>	-	23699479	23698977	503	1	15269
KLK5	-	23724466	23714749	9718	6	5611
KLK6	-	23741119	23730078	11042	7	6799
KLK7	-	23755340	23747919	7422	6	12113
KLK8	-	23773148	23767454	5695	6	810
KLK9	-	23781080	23773959	7122	5	4635
KLK10	-	23791472	23785716	5757	6	2204
KLK11	-	23799480	23793677	5804	6	1057
KLK12	-	23806338	23800538	5801	6	21314
KLK13	-	23836557	23827653	8905	5	12206
KLK14	-	23855692	23848764	6929	8	14272
CAG <sup>b</sup>	-	23876037	23869965	6073	3	20317
SIGLEC9	+	23896355	23901757	5403	7	

<sup>a</sup>Coordinates for  $\Psi$ PPIA are provisional.

<sup>b</sup>CAG is also known as LOC90353.

in the promoters and enhancers of genes, as well as in introns and in the untranslated regions of mRNAs. Polymerase chain reaction (PCR) analysis of two clusters of these elements indicates that they are polymorphic, and thus they can be useful tools in linkage analysis and DNA fingerprinting. Interestingly, one of the clusters was found to extend from the last part of exon 3 of the *KLK14* gene. Our preliminary data show that the distribution of the different alleles of these minisatellites might be associated with malignancy [40].

## 5. Structural Features of the Human Tissue Kallikrein Genes and Proteins

Extensive analyses over the last few years have led to the identification of many common structural features of kallikreins. Some of these features are shared with other members of the S1 family of serine proteases (see

following). Other features, however, are unique to certain kallikreins. As mentioned above, some of the kallikrein-specific features are conserved among species (e.g., all kallikreins have five coding exons, and only one member in each species has true kininogenase activity). Human tissue kallikrein gene lengths range from 4 to 10 kb, with most of the differences attributed to introns.

### 5.1. COMMON STRUCTURAL FEATURES

The common structural features of kallikreins can be summarized as follows (see also Table 3) [34, 42–44]. First, all genes possess five coding exons (except for a *KLK4* variant, which has four exons), and most of them have one or two extra 5' untranslated exons. The first coding exon always contains a 5' untranslated region, followed by the methionine start codon, located 37–88 bp away from the end of the exon. The stop codon is always located 150–189 bp from the beginning of the last coding exon. Second, exon sizes are very similar or identical. Third, the intron phases of the coding exons (i.e., the position where the intron starts in relation to the last codon of the previous exon) are conserved in all genes. The pattern of the intron phase is always I-II-I-0. Fourth, the positions of the residues of the catalytic triad of serine proteases are conserved, with the histidine always occurring near the end of the second coding exon, the aspartate at the middle of the third coding exon, and the serine residue at the beginning of the fifth coding exon. Fifth, all kallikrein proteins are predicted to be synthesized as pre-pro-peptides, with a signal peptide of 16–57 amino acids at the N terminus, followed by an activation peptide of about three to nine amino acids, followed by the enzymatically active (mature) protein (223–252 amino acids). Sixth, the amino acid of the substrate-binding pocket is either aspartate (11 kallikreins) or glutamate (1 kallikrein), indicating trypsin-like specificity (12 kallikreins), or another amino acid (probably conferring chymotryptic or other activity), as is the case with hK3 (serine), hK7 (asparagine), and hK9 (glycine). Seventh, in addition to the conservation of the catalytic amino acid triad, seven additional protein motifs were also found to be highly conserved in kallikreins [44]. Eighth, most, if not all, kallikrein genes are under steroid hormone regulation. Ninth, all proteins contain 10–12 cysteine residues, which will form five (in hK1, hK2, hK3, and hK13) or six (in all other kallikreins) disulphide bonds. The positions of the cysteine residues are also fully conserved. Finally, classical or variant polyadenylation signals have been found 10–20 bases away from the poly-A tail of all kallikrein mRNAs. All three classical kallikreins have the same variant polyadenylation signal AGTAAA [5, 33, 45–47]. Multiple alignments of all kallikrein proteins have been published previously [43].

TABLE 3  
SUMMARY OF HUMAN TISSUE KALLIKREIN PROTEIN CHARACTERISTICS

Protein name	pI <sup>a</sup>	MW <sup>b</sup>	Full length <sup>c</sup>	Signal peptide <sup>c</sup>	Activation peptide <sup>c</sup>	Mature protein <sup>c</sup>	Cysteines <sup>d</sup>	Substrate specificity <sup>e</sup>
hK1	4.62	28.9	262	17	7	238	10	Trypsin-like
hK2	6.44	28.7	261	17	7	237	10	Trypsin-like
hK3	6.78	28.8	261	17	7	237	10	Chymotrypsin-like
hK4	4.71	27.0	254	26	4	224	12	Trypsin-like
hK5	8.64	32.0	293	57	9	227	12	Trypsin-like
hK6	7.15	26.8	244	16	5	223	12	Trypsin-like
hK7	8.82	27.5	253	22	7	224	12	Chymotrypsin-like
hK8	8.61	28.5	260	28	4	228	12 <sup>d</sup>	Trypsin-like
hK9	7.10	27.5	250	19	3	229	12	Chymotrypsin-like
hK10	8.95	30.1	276	33	9	234	12	Trypsin-like
hK11	8.80	27.5	250	18	3	229	12 <sup>f</sup>	Trypsin-like
hK12	7.57	26.7	248	17	4	227	12	Trypsin-like
hK13	8.78	30.6	277	20	5	252	10	Trypsin-like
hK14	9.30	27.5	251	18	6	227	12	Trypsin-like
hK15	8.27	28.1	256	16	5	235	12	Trypsin-like

<sup>a</sup>Isoelectric point.

<sup>b</sup>Molecular weight of the full-length protein in kilodaltons, excluding any post-translational modifications.

<sup>c</sup>Number of amino acids.

<sup>d</sup>The number of cysteine residues in the mature enzyme.

<sup>e</sup>Some substrate specificities have not been experimentally verified.

<sup>f</sup>Has an extra "C" residue in a nonconserved position.

## 5.2. THREE-DIMENSIONAL STRUCTURE

The crystal structure has been revealed for some rodent kallikreins. The three-dimensional structure of a horse orthologue of the human hK3 has also been reported [28]. In contrast, hK1 and hK6 are the only human kallikreins for which crystal structures have been determined [48–50]. Most of the discussion in this section is derived from comparative model building of the human kallikrein proteins.

Kallikreins can be roughly divided into two categories, the classical kallikreins (hK1, hK2, and hK3) and the “new” kallikreins. The new kallikreins appear to be unique in their three-dimensional structure and share some features with trypsins and other features with the classical kallikreins. Comparative protein models show that the pattern of hydrophobic side-chain packing in the protein core is nearly identical in all human kallikreins, and the observed differences occur within the solvent-exposed loop segments.

An 11-amino acid residue insertion relative to the trypsin sequences in loop E (residues 91–103 in the bovine chymotrypsinogen consensus numbering), also known as “the kallikrein loop,” is a unique feature of the three classical kallikreins. Loop E is located between the fifth (residues 81–90) and the sixth strand (residues 104–108), and loop G between the seventh and the eighth strand (residues 156–163). None of the new human kallikreins contains this loop in its entirety. Loop E in hK10 is longest, with an eight-residue-long insertion relative to the trypsin sequences. Loop E overhangs the substrate-binding groove on the surface of the protease molecule, and its length and sequence can directly influence substrate recognition.

The *KLK15* gene is particularly interesting, as it lies between two classical tissue kallikrein genes, *KLK1* and *KLK3* [51], yet the sequence and structure of hK15, with six disulphide bonds and no insertion in the so-called kallikrein loop (E), clearly place it among the new kallikreins. Moreover, in loop G, hK15 has an eight-residue insertion [51] that is not found in any other kallikrein. In the three-dimensional structure, the extended loop G lies on the opposite side of the active site relative to loop E, and although it is more distant to the substrate-binding groove, loop G may also participate in substrate and inhibitor recognition.

## 6. Sequence Variations of Human Kallikrein Genes

Sequence changes, including polymorphisms and mutations, are clinically important. In addition to medicolegal applications, they can also be indicators for susceptibility and prognosis for different malignancies [52]. *KLK3* is the most extensively studied kallikrein in this respect. Comparison of the

published mRNA sequences of *KLK3* revealed infrequent and inconsistent sequence variations. Baffa *et al.* found no evidence of mutations in the *KLK3* mRNA sequence in prostate cancer compared to matched normal tissues from the same patient [53]. Similarly, no mutations were found in the coding portion of the *KLK3* gene in breast cancer tissues and cell lines, with the exception of a polymorphism in exon 2 in some breast tumors [54]. Three distinct forms of *KLK1* mRNA, differing in one or two amino acid substitutions, were identified in different tissues [1, 55, 56]. Experimental evidence, however, indicates that the protein products of these variants have no difference in their protein activity [57]. Probably the most polymorphic sequence of *KLK4* is that deposited by Hu *et al.* [41]. In addition to a large insertion in the 3' untranslated region, there are 18 differences between their sequence and those deposited by others. These probable polymorphisms will affect the derived amino acid product [41]. We have recently identified a germline single nucleotide variation in exon 3 of the *KLK10* gene that will change the amino acid from alanine to serine. This polymorphism is less prevalent in prostate cancer patients in comparison to control subjects [58]. Also, four other polymorphisms were identified in exon 4 of the same gene.

Within a 5.8-kb promoter/enhancer region of *KLK3*, 16 different mutational hotspots (appearing more than once in nine tumors) were found in breast cancer [54]. A single nucleotide variation (G → A) was identified at position -158 within androgen response element 1 (ARE-1). Univariate Cox regression modeling showed a 28% reduction in the risk of death in patients with homozygous G genotype compared to those with homozygous A [59].

In general, the examination of sequence variation within the 300-kb kallikrein locus has not been performed in detail, and it is therefore possible that inactivating mutations within the coding region of kallikrein genes exist but await discovery. Kallikrein gene inactivation in human diseases would likely provide clues for the physiological functions of these diseases.

## **7. The Tissue Kallikreins in the Context of Other Serine Proteases in the Human Genome**

Proteases are enzymes that cleave proteins by hydrolyzing peptide bonds. On the basis of their catalytic mechanisms, they can be classified into five main types: proteases that have an activated cysteine residue (cysteine proteases), an activated aspartate (aspartate proteases), a metal ion (metalloproteases), or an activated threonine (threonine proteases), and proteases with an active serine (serine proteases). Within each type, enzymes are separated into “clans” (also referred to as “superfamilies”) based on evidence of evolutionary relationship [60, 61] from the linear order of

catalytic site residues and the tertiary structure, in addition to distinctive aspects of catalytic activity such as specificity or inhibitor sensitivity. Each clan is given a two-letter identifier, of which the first letter is an abbreviation for the catalytic type. Next, proteins are classified into families (each denoted by a unique number) and subfamilies (denoted by another letter) based on sequence similarity to a chosen type example for that family.

### 7.1. GENOMIC OVERVIEW OF SERINE PROTEASES

According to our recent survey, and studies by others, there are approximately 500 confirmed, nonredundant proteases in the human genome, including nonpeptidase homologues [62–65]. This represents about 2% of all gene products in humans [60]. This number increases to about 700 when the “predicted” genes and proteins are included. Approximate figures indicate that proteases are distributed as follows: 4% are aspartate proteases, 26% are cysteine proteases, 34% are metalloproteases, 5% are threonine proteases, and 31% are serine proteases.

Serine proteases (SP) are a family of enzymes that use a uniquely activated serine residue in the substrate-binding pocket to catalytically hydrolyze peptide bonds [66]. SP carry out a diverse array of physiological functions, of which the best known are digestion, blood clotting, fibrinolysis, fertilization, and complement activation during immune responses [67]. They have also been shown to be abnormally expressed in many diseases including cancer, arthritis, and emphysema [42, 43, 67–70].

Out of the estimated 500 proteases in the human genome, 31% are predicted to be serine proteases [71]. This large family includes the digestive enzymes (e.g., trypsin, chymotrypsin), the kringle domain-containing growth factors (e.g., tissue plasminogen activator), some of the blood clotting factors, and the kallikreins. In terms of absolute numbers, a total of 150 serine proteases have been identified in the human genome and are distributed in all chromosomes except 18 and Y [62]. The density of serine proteases varies from just one to two genes in most chromosomes to up to 23 genes on chromosome 19. Most SP are localized sporadically, and relatively few clusters exist. Kallikreins represent the largest cluster of serine proteases in the human genome.

### 7.2. STRUCTURAL AND SEQUENCE ANALYSIS

From a structural point of view, kallikreins belong to the serine protease family of enzymes. The essential features of serine proteases that are preserved in the kallikreins can be summarized as follows: only one serine residue of the protein is catalytically active; two residues, a histidine and an

aspartate, are always associated with the activated serine in the catalytic site, forming together what is known as the “catalytic triad” of serine proteases; each of the catalytic triad residues is surrounded by a highly conserved motif [the motif GDSGGP surrounds serine, TAAHC surrounds histidine, and DIMLL surrounds aspartate residues [60]]; the active serine is situated in an internal pocket, and the aspartate and histidine residues are closely located in the three-dimensional structure; the catalytically essential histidine and serine are almost immediately adjacent to their exon boundaries; they are initially produced in a “zymogen” form; they exhibit a high degree of sequence similarity; and they contain most of the 29 amino acids that are reported by Dayhoff to be invariable in many species [72].

Kallikreins belong to the S1 family (also known as the trypsin family), of clan SA of serine proteases. This is supported by sequence, structural, and phylogenetic analyses. We have recently performed detailed analyses for 79 protein sequences representing this family, according to the latest information from the Human Genome Project and other databases [63]. Our results show that seven residues are absolutely conserved in this family. An additional 15 showed almost complete conservation (>95%), and in total, 48 residues were found to be more than 80% conserved, and 87 residues were found to display greater than 50% conservation. Conserved residues tended to group together, likely representing certain necessary structural or functional domain elements. This conclusion is supported by the fact that in most cases of substitution, a residue of similar character (i.e., size, hydrophobicity, or polarity) was inserted. This implies that the overall character of the local region is conserved for proper function, more so than some of the individual amino acid identities. In addition to the conserved motifs around the residues of the catalytic triad, we recently identified 32 other highly conserved amino acid motifs in the S1A family of SP, including tissue kallikreins [63]. The biological significance of these motifs has yet to be determined. Multiple alignment showed the presence of a conserved domain (R/K)(I/V)(V/I)(G/N) at the N-terminal cleavage site of the zymogen (proenzyme) end of most members of this family. Most enzymes are cleaved after an Arg or Lys, indicating the need for a trypsin-like enzyme for activation. In the case of trypsin, cleavage occurs between residues Lys<sup>15</sup> and Ile<sup>16</sup> (chymotrypsinogen numbering). After cleavage Ile<sup>16</sup> forms the new N terminus of the protein, and Asp<sup>194</sup> rotates to interact with it. This rotation, and the resulting salt bridge, produces a conformational change that completes the formation of the oxyanion hole and the substrate binding pocket, both of which are necessary for proper catalytic activity. Certain sequences did not display conservation of this trypsin cleavage site, with substitutions at either the 15th or 16th positions (e.g., the granzymes). These substitutions likely result in either cleavage by a protease with different specificity or no cleavage at all. The presence of Asp at position 189 indicates that most members of



the S1A family will have a trypsin-like specificity. In chymotrypsin and chymotrypsin-like proteases (e.g., hK3), there is a serine at this position.

Structurally conserved regions usually remain conserved in all members of the family and are usually composed of secondary structure elements, the immediate active site, and other essential structural framework residues of the molecule. For instance, Ser<sup>214</sup> in chymotrypsin-like proteases contributes to the S1 binding pocket and appears to be a fourth member of a catalytic tetrad [73]. Between these conserved elements are highly variable stretches (also called variable regions). These are almost always loops that lie on the external surface of the protein and that contain all additions and deletions between different protein sequences. The former regions (structurally conserved regions) have been successfully used as the basis for predicting the three-dimensional structure of newly identified SP based on information on existing members [74]. The latter (variable regions) are important for studying the evolutionary history of SP.

Certain residues with a variable degree of conservation can be investigated for their usefulness as “evolutionary markers” that can provide insight into the history of each enzyme family or clan and allow comparative analysis with other families or clans. Krem and Di Cera [75] identified several such markers with proven evolutionary usefulness. In addition to the use of these markers for rooting the phylogenetic trees, attempts were made to classify serine proteases into functional groups based on these markers or their coding sequences.

Serine proteases exhibit preference for hydrolysis of peptide bonds adjacent to a particular class of amino acids. In the trypsin-like group, the protease cleaves peptide bonds following basic amino acids such as arginine or lysine, as it has an aspartate (or glutamate) in the substrate-binding pocket, which can form a strong electrostatic bond with these residues. The chymotrypsin-like proteases have a nonpolar substrate-binding pocket and thus require an aromatic or bulky nonpolar amino acid such as tryptophan, phenylalanine, tyrosine, or leucine. The elastase-like enzymes, however, have bulky amino acids (valine or threonine) in their binding pockets, thus requiring small hydrophobic residues, such as alanine [76].

Activation reactions catalyzed by serine proteases (including kallikreins) are an example of “limited proteolysis” in which the hydrolysis is limited to one or two particular peptide bonds. Hydrolysis of peptide bonds starts with the oxygen atom of the hydroxyl group of the serine residue that attacks the carbonyl carbon atom of the susceptible peptide bond. At the same time, the serine transfers a proton first to the histidine residue of the catalytic triad and then to the nitrogen atom of the susceptible peptide bond, which is then cleaved and released. The other part of the substrate is now covalently bound to the serine by an ester bond. The charge that develops at this stage is partially neutralized by the third (aspartate) residue of the catalytic triad. This process is followed by “deacylation,” in which the histidine draws a

proton away from a water molecule and the hydroxyl ion attacks the carbonyl carbon atom of the acyl group that was attached to the serine. The histidine then donates a proton to the oxygen atom of the serine, which will then release the acid component of the substrate.

## **8. Tissue Expression and Cellular Localization of the Kallikrein Genes**

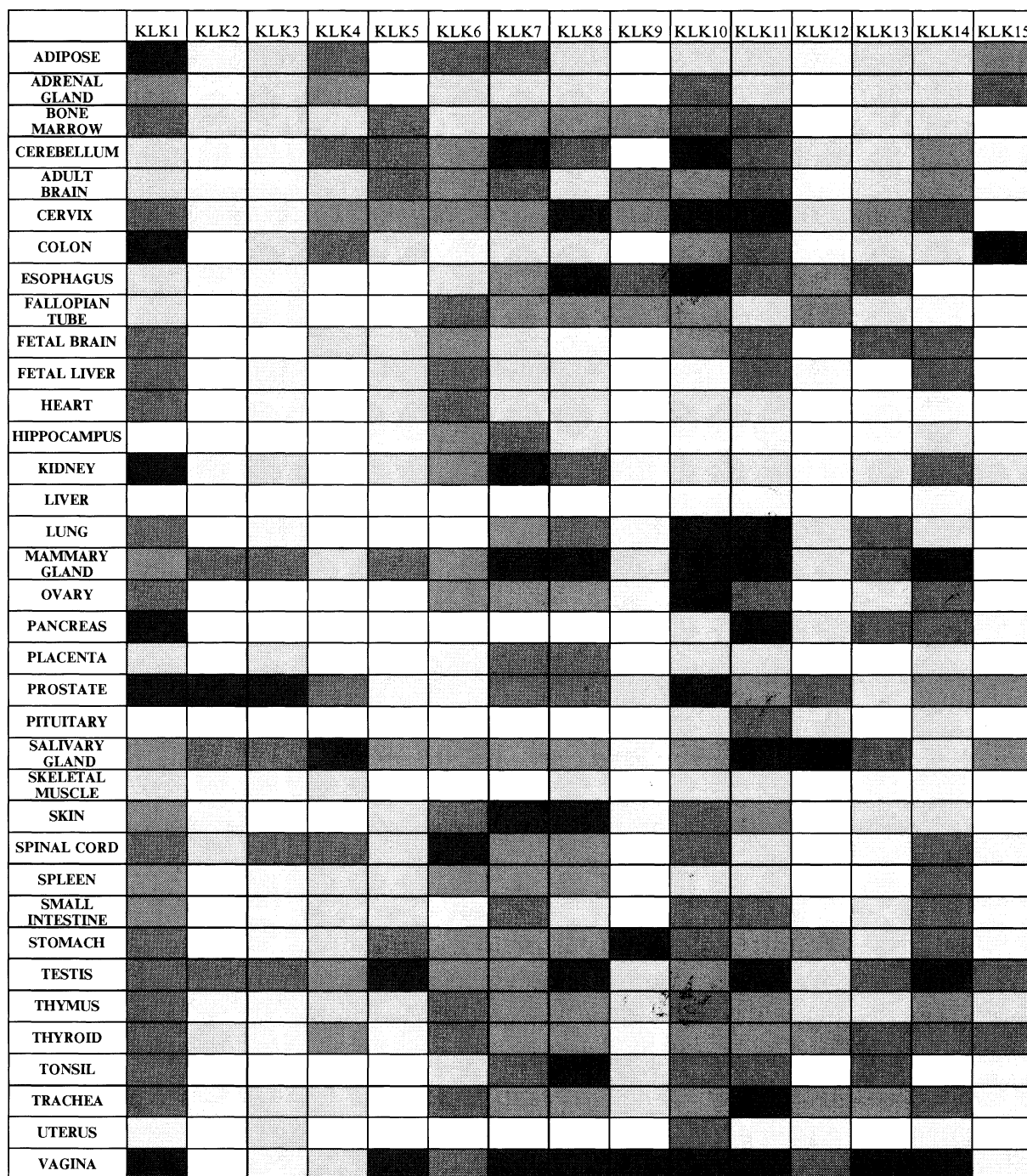
### 8.1. OVERVIEW

Many kallikreins are transcribed predominantly in only a few tissues, as indicated by Northern blotting. By using the more sensitive reverse transcriptase (RT)–PCR technique, kallikreins were found to be expressed at lower amounts in several other tissues. Many kallikreins are expressed in the salivary gland—the tissue in which most of the rodent kallikreins are expressed. In addition, several kallikreins were also found in the central nervous system and endocrine-related tissues such as the prostate, breast, and testis. A more detailed review of the expression of kallikreins in different tissues by various techniques can be found elsewhere [44]. A global view of kallikrein expression in 36 different tissues, as determined by RT-PCR, is presented in Fig. 2.

### 8.2. IMMUNOHISTOCHEMICAL EXPRESSION OF HUMAN TISSUE KALLIKREINS

Most studies use quantitative methods such as quantitative RT-PCR to reveal the expression of human tissue kallikreins in benign and malignant tissues. The recent development by our group of monoclonal and polyclonal antibodies against many kallikrein proteins has helped in defining their distribution in tissue extracts [77–79]. Furthermore, using the streptavidin–biotin method with monoclonal and polyclonal antibodies, we have already studied the immunohistochemical expression (IE) of seven hKs (hK5, hK6, hK7, hK10, hK11, hK13, and hK14) in several normal human tissues, as well as in the corresponding malignant tissues [6, 80–85, and our unpublished data). Different antibodies used for each kallikrein (polyclonal and monoclonal) revealed a similar immunostaining pattern in many tissues. The IE was always cytoplasmic and in some tissues displayed a characteristic immunostaining pattern that was membranous, droplet-like, supranuclear, subnuclear, and luminal.

Comparison of the IE patterns of a few studied kallikreins in different tissues revealed no major differences, indicating that they may share a common mode of regulation. It is worth mentioning that our results concerning the IE of the studied hKs in different normal human tissues correspond fairly



SEMIQUANTITATIVE EXPRESSION SCORING SYSTEM					
Very High	High	Moderate	Low	Very Low	Nothing

FIG. 2. Expression map of human tissue kallikreins in a variety of tissues, as determined by reverse transcriptase polymerase chain reaction. The relative semiquantitative expression levels for each gene are indicated. (See Color Insert.)

well with other data based on ELISA assays and RT-PCR. According to these studies, except for *KLK2* and *KLK3*, none of the remaining *KLKs* is tissue-specific, although certain genes are preferentially expressed in some organs [31, 86–88]. Many hKs were immunohistochemically revealed in a variety of tissues, indicating that no protein is tissue specific. Immunohistochemistry has advantages over other methods, as it defines the protein distribution in different cell types independent from its quantity in the tissue. A tissue may therefore immunohistochemically express a kallikrein and yet yield negative results using a quantitative method. This likely explains why we did not find any immunohistochemical difference in the tissue expression among the different hKs, whereas other methods showed tissue preferences for each hK.

In recent RT-PCR studies, many kallikreins have been proposed as new biomarkers for malignancies other than prostate cancer. Breast, ovarian, and testicular cancers are the most studied. Certain kallikreins were found to be differentially expressed in various malignancies (up- or downregulated), and the increase or decrease of their expression may be associated with prognosis [43, 58, 70, 89–94]. We have immunohistochemically evaluated some kallikreins in malignant diseases, including two series of prostate and renal cell carcinoma, and have examined their prognostic values [84, 85].

In malignancy, glandular epithelia constitute the main kallikrein immunoe-expression sites, and staining of their secretions indicating that these proteases are secreted. Similar to the IE pattern in normal glandular tissues, all hKs are expressed in adenocarcinomas. In the future, it is clearly worthwhile to study the relation of the IE of all hKs with prognosis of several malignancies and to correlate these results with those obtained by other methods.

In Figs. 3–5, we present immunohistochemical data for various kallikreins. For more discussion, please refer to our detailed publications [80–85].

Regarding immunohistochemical localization, hK4 appears to be a notable exception. Recently, Xi *et al.* suggested that hK4 is a predominantly nuclear protein that is overexpressed in prostate cancer [95]. It appears that the vast majority of *KLK4* mRNA lacks exon 1, which codes for the signal peptide. These preliminary data need to be reproduced.

## 9. Regulation of Kallikrein Activity

### 9.1. AT THE MRNA LEVEL

Promoter analysis and hormonal stimulation experiments allowed us to obtain insights into the mechanisms that regulate expression of the human kallikrein genes. In addition to *KLK3* and *KLK2*, and more recently *KLK10*, no other kallikrein gene promoter has been functionally tested.

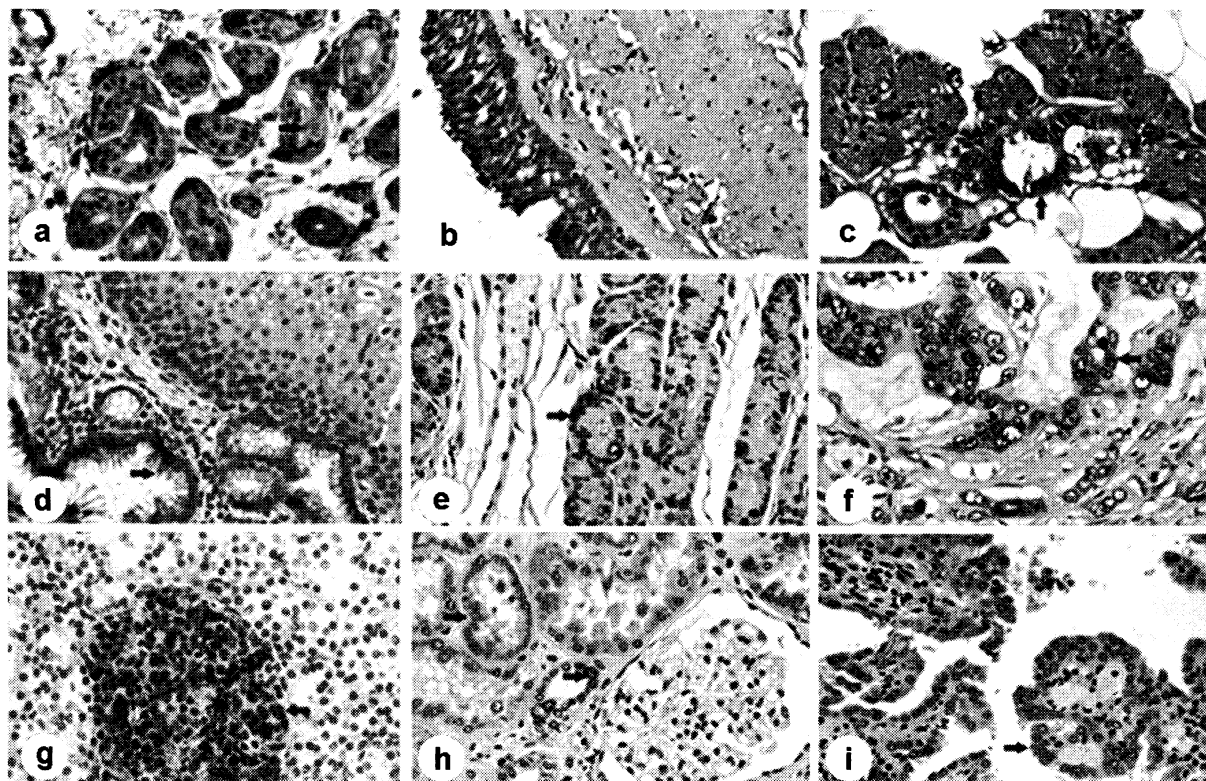


FIG. 3. Immunohistochemical expression of (a) hK7 by the epithelium of eccrine glands of the skin (monoclonal antibody, clone 73.2), (b) hK13 by the epithelium of the bronchus (monoclonal antibody, clone IIC1), (c) hK5 by the ductal epithelium of the parotid gland (polyclonal antibody), (d) hK7 by the esophageal glands (monoclonal antibody, clone 73.2), (e) hK13 by the gastric mucosa (monoclonal antibody, clone 2-17), (f) hK6 by the large intestine mucosa (polyclonal antibody), (g) hK10 in an islet of Langerhans in the pancreas (monoclonal antibody, clone 5D3), (h) hK11 by the epithelium of the urinary tubuli (monoclonal antibody), (i) hK11 by a papillary renal cell carcinoma (monoclonal antibody). (See Color Insert.)

TATA box variants are found in the three classical kallikreins. *KLK1* has the variant TTTAAA, whereas *KLK2* and *KLK3* share another variant, TTTATA. [5, 33, 45-47]. In addition, two AREs have been identified and experimentally verified [96]. Another ARE was mapped in the far upstream enhancer region of the gene and shown to be functional and tissue specific [97, 98]. More recently, five additional low-affinity AREs have been identified close to ARE-III [99], and three distinct regions surrounding ARE-III were found to bind ubiquitous and cell specific proteins. A functional ARE was also identified in the *KLK2* promoter [100]. Interestingly, a negative regulatory element was also found between -468 and -323 of *KLK2* [100], and another ARE was identified between -3819 and -3805 of the *KLK2* promoter [101].

Apart from *KLK1*, *KLK2*, and *KLK3*, no obvious TATA boxes were found in the promoter of other kallikreins. Two major obstacles exist in predicting the promoter response elements: the inaccurate localization of

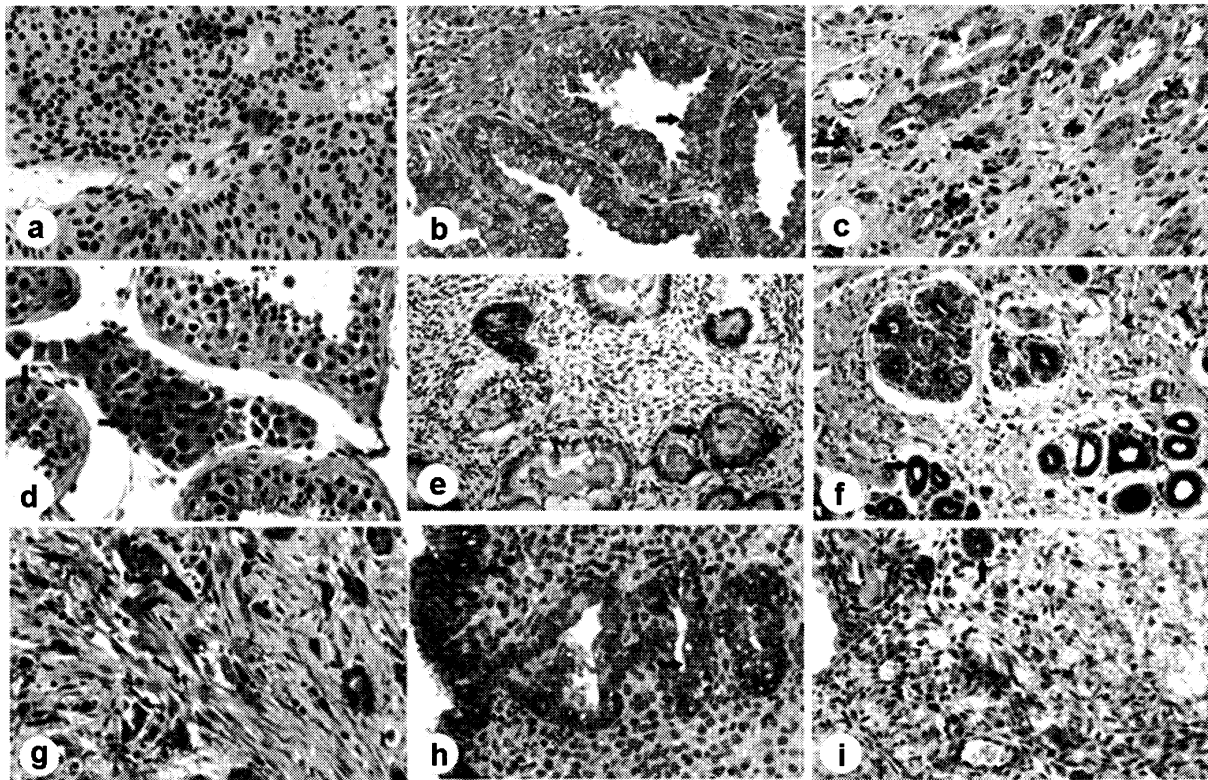


FIG. 4. Immunohistochemical expression of: (a) hK10 by a low-grade urothelial carcinoma (monoclonal antibody, clone 5D3), (b) hK11 by the secretory epithelium of the prostate gland (polyclonal antibody), (c) hK11 by a Gleason score 6 prostate carcinoma (polyclonal antibody), (d) hK14 by the spermatogenic epithelium and the stromal Leydig cells in the testis (polyclonal antibody), (e) hK10 by epithelial elements in a testicular immature teratoma (monoclonal antibody, clone 5D3), (f) hK14 by lobuloalveolar structures of the breast (polyclonal antibody), (g) hK14 by a ductal breast carcinoma, grade II (polyclonal antibody), (h) hK13 by the glandular epithelium of the endometrium (polyclonal antibody), (i) hK14 by luteinized stromal cells of the ovary (polyclonal antibody). (See Color Insert.)

the transcription start site and the presence of more than one splice variant with more than one transcriptional start site. Recently, by using EST analysis alone, Grimwood *et al.* extended the 5'-end of many published mRNAs from chromosome 19 by more than 50 bp [3].

## 9.2. AT THE PROTEIN LEVEL

There are different mechanisms for controlling serine protease activity by which unwanted activation is avoided and precise spatial and temporal regulation of the proteolytic activity is achieved. One important mechanism is by producing kallikreins in an inactive “proenzyme” (or zymogen) form, which will be activated as necessary. The N-terminal extension of the mature enzyme, or the “prosegment,” sterically blocks the active site and thus prevents binding of substrates. It is also possibly implicated in folding, stability, and intracellular sorting of the zymogen. For more detailed

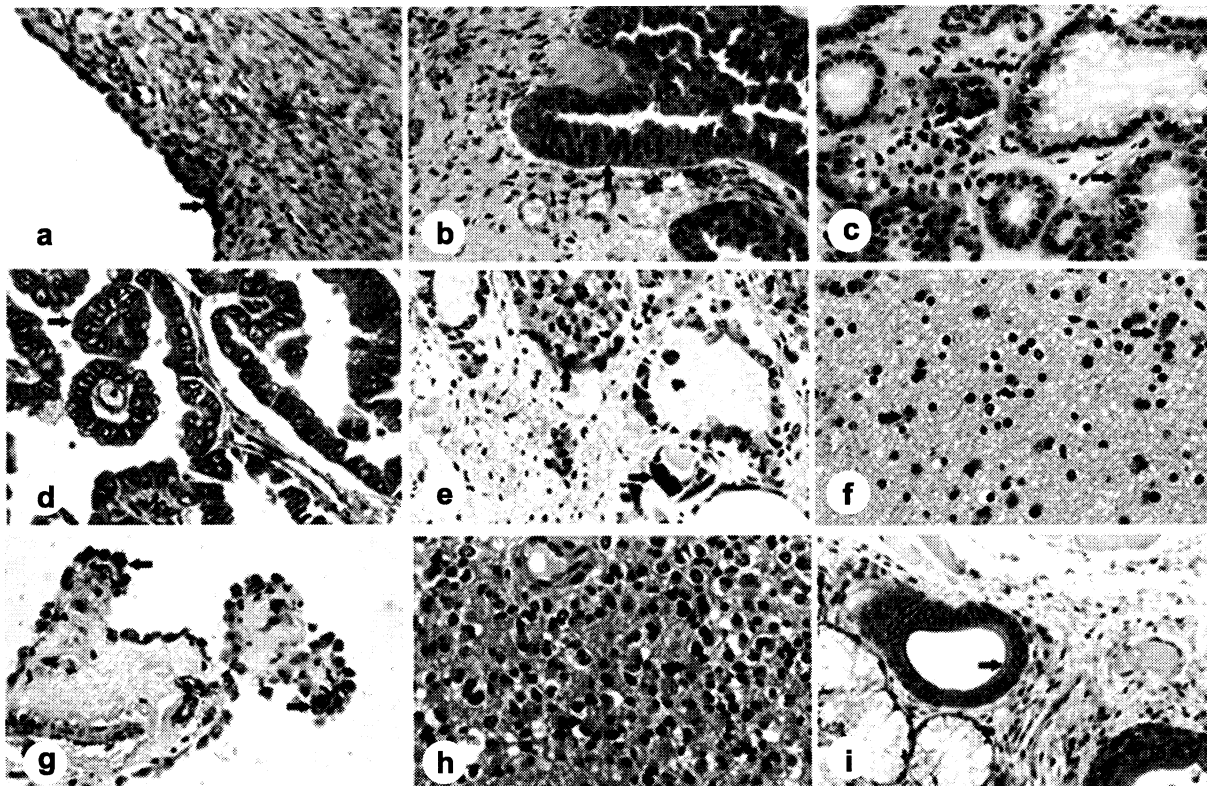


FIG. 5. Immunohistochemical expression of (a) hK14 by the ovarian surface epithelium (polyclonal antibody), (b) hK14 by a cystadenocarcinoma of the ovary (polyclonal antibody), (c) hK10 by hyperplastic follicles of the thyroid gland (monoclonal antibody, clone 5D3), (d) hK6 by a papillary thyroid carcinoma (polyclonal antibody), (e) hK13 by endocrine cells in the pituitary gland (monoclonal antibody, clone 2-17), (f) hK10 by glial cells in the brain (monoclonal antibody, clone 5D3), (g) hK13 by the choroid plexus epithelium (monoclonal antibody, clone 2-17), (h) hK5 by a glioma (monoclonal antibody, clone 6.10), (i) hK7 by the ducts of the submucosal glands of the tonsils (monoclonal antibody, clone 85.2). (See Color Insert.)

discussion, see the recent review by Khan and James [102]. The activation of the zymogen can occur intracellularly (i.e., in the trans-Golgi apparatus or in the secretory granules) or extracellularly after secretion, and it can be autolytic or dependent on the activity of another enzyme (see following). Interestingly, all of the “proforms” of the kallikrein enzymes, with the exception of hK4, are predicted to be activated by cleavage at the C-terminal end of either arginine or lysine (the preferred trypsin cleavage site), indicating that they need an enzyme with trypsin-like specificity for their activation. This observation has been experimentally demonstrated for some kallikreins. For example, hK5 and hK7 can be converted to the active enzyme by trypsin treatment [103, 104], and hK11 can be activated by enterokinase. Autoactivation is a common phenomenon among kallikreins—hK2, though not hK3, is capable of autoactivation [105]. hK3 has chymotryptic activity but it needs a trypsin-like activating enzyme. hK4 is also autoactivated

during the refolding process [106], and there is evidence that hK6 is also capable of autoactivation [107]. hK13 is also autoactivated on secretion (G. Sotiropoulou, personal communication). This autoactivation can be explained by the finding that many kallikreins have trypsin-like substrate activity and that the same type of activity is needed for their activation.

Proteolytic activation is irreversible. Hence, other means of switching off the activity of these enzymes are needed. Once activated, serine proteases are controlled by ubiquitous endogenous inhibitors. Laskowski and Qasim divide all known inhibitors into two categories: inhibitors devoid of significant class specificity, and class-specific inhibitors [108]. The former includes proteins of the  $\alpha_2$ -macroglobulin family, which bind proteases through a molecular trap mechanism and inhibit them by steric hindrance [109]. With respect to specific serine protease inhibitors, at least 23 structurally distinct families are currently known, including the Kunitz, soybean trypsin inhibitors–Kunitz, Kazal, and hirudin families, as well as the serpins (serine proteinase inhibitors) [108]. Many of the specific inhibitors are capable of inhibiting the same serine protease, and the same inhibitor may inhibit several serine proteases [108]. Some molecular complexes of kallikreins with protease inhibitors have clinical applicability because they can improve the diagnostic sensitivity or specificity of cancer biomarkers such as PSA [110].

The majority of hK3 (PSA) in the prostate and seminal plasma is in its free form, with less than 5% complexed with protein C inhibitor [111]. In serum, the majority of hK3 binds to protease inhibitors, and only 15%–25% is in the free form. The hK3– $\alpha_1$ -antichymotrypsin complex constitutes 70%–85% of total serum hK3, with the hK3– $\alpha_2$ -macroglobulin and hK3– $\alpha_1$ -antitrypsin complexes representing 15% and 3%, respectively [112–114].

hK2 has been shown to complex with protease C inhibitor both in *in vitro* studies and in seminal plasma [115]. In serum, most of hK2 is in the free form, with only a small amount complexed with  $\alpha_1$ -antichymotrypsin [116, 117].  $\alpha_2$ -macroglobulin [118], and antithrombin III [119] have also been shown to be able to bind hK2. hK2 was also found to be bound to  $\alpha_2$ -antiplasmin and plasminogen activator inhibitor 1 [120]. More recently, hK5 was found to form complexes with  $\alpha_1$ -antitrypsin [121],  $\alpha_1$ -antichymotrypsin was identified as an inhibitor for hK6 [122], and hK13 was reported to form complexes with  $\alpha_1$ -antichymotrypsin [123]. Another mechanism for controlling the activity of proteases is by internal cleavage and subsequent degradation. Self-digestion is reported for hK7 [104]. Around 30% of hK3 in seminal plasma is inactivated by internal cleavage between lysine 145 and lysine 146 [124], and about 25% of hK2 was also found to be internally cleaved between amino acids 145–146 (Arg-Ser) [125]. Other internal cleavage sites have also been identified for hK2 and hK3, but the enzymes responsible for such cleavages are not yet known.



### 9.3. LOCUS CONTROL OF KALLIKREIN EXPRESSION

The coexpression of many kallikreins in the same tissues and the parallel differential regulation of groups of kallikreins in pathological conditions (e.g., the upregulation of seven kallikreins in ovarian cancer [126]) raise the possibility of the existence of a common mechanism that controls the expression of groups of kallikrein genes in a cluster known as a locus control region (LCR). Added to this are the relatively short distances between adjacent kallikreins [which could be as short as the 1.5 kb between *KLK1* and *KLK15* [34]] and the absence of classic promoter sequences, as shown by prediction analysis, in all kallikreins except *KLK2* and *KLK33*. Clustering of coexpressed homologous genes could be explained by the evolutionary history of the genomic region. The probable mechanism in this case would include local duplication and divergence of amplified copies, resulting in an array of paralogues that may retain common regulatory elements [127].

Previous studies have shown that two sequence elements were essential for initiating DNA replication of an adjacent group of beta globin genes: the initiation region and the LCR, residing 50 kb upstream of the initiation region. The beta globin LCR is located 6–20 kb upstream of a cluster of five functional globin genes. It consists of five DNase hypersensitive sites and numerous binding sites for transcription factors. LCRs are operationally defined by their ability to enhance the expression of linked genes to physiological levels in a tissue-specific and copy number-dependent manner [128]. Although their composition and locations relative to their cognate genes are different, LCRs have been described in a broad spectrum of mammalian gene systems, indicating that they play an important role in the control of eukaryotic gene expression. Other intergenic sequences, such as domain boundaries or barriers, and chromatin architecture might also be involved. Acquisition of knowledge about these processes is a key step toward the understanding of the role of kallikreins in normal physiology and pathobiology.

Another proposed regulatory mechanism is gene potentiation, which is the process of opening a chromatin domain that will render genes accessible to the various factors required for their expression. The formation of an open chromatin structure is central to the establishment of cell fate and tissue-specific gene expression. Many eukaryotic genes are organized into functional chromatin domains. This facilitates their coordinated regulation during development [129]. The ability of individual cells to regulate the genes contained within such chromatin domains is of paramount importance for their differentiation. Perturbations in chromatin structure can act both locally, to alter the accessibility of *trans*-acting factors to *cis*-regulatory elements, and globally, to affect the opening and closing of entire chromatin domains [130]. The potentiated state of a gene can also be influenced by alterations in

the local chromatin environment. For example, many eukaryotic genes are differentially expressed by altering their methylation status. These genes are largely unmethylated in cells where they are transcribed but fully methylated in all nonexpressing cells [131]. Histone acetylation also acts on the local gene environment during the transition from the 30-nm fiber to a more open structure that can be linked to a 10-nm fiber, stabilizing the more relaxed open structure [132]. It has also been postulated that DNA methylation patterns may serve to modulate histone acetylation, thereby maintaining local chromatin states. Both DNA demethylation and histone acetylation render increased accessibility of ubiquitous and tissue-specific *trans*-acting factors to *cis*-regulatory elements, facilitating transcriptional activation [133].

#### 9.4. EPIGENETIC REGULATION OF KALLIKREIN GENE EXPRESSION

In human cancers, numerous mechanisms may contribute to loss of tumor suppressor gene function, including homozygous deletions, allelic loss in combination with mutations, abnormal splicing, and CpG island methylation [134]. Methylation contributes to inactivation of numerous genes, including the cell cycle regulator *p16* [135], the growth suppressor *ER* [136], the epithelial cadherin *E-cadherin* [137], the DNA repair gene *MGMT* [138], the Ras-associated domain family 1A gene *RASSF1A* [139], the angiogenesis inhibitor *THBS1* [140], and the metastasis suppressor *TIMP3* [141]. In addition, hK10 can also be inactivated by CpG island hypermethylation in breast cancer [142] and acute lymphoblastic leukemia [143].

The physiological function of hK10 is still unclear. However, recent data indicate that *KLK10* may have a tumor suppressor function, based on its downregulation in breast and prostate cancer cell lines and the finding that overexpression of *KLK10* in nude mice can suppress tumor formation [144, 145]. The expression of the protein in certain tissues and its homology to other members of the kallikrein family should provide a starting point in the search for physiologically relevant substrates.

This putative tumor suppressor activity prompted us to speculate that this gene may be a target for either somatic mutations or hypermethylation, in analogy to other tumor suppressor genes that are inactivated by mutations or methylation and that thereby predispose to cancer development or progression. A previous study from our laboratory [58] examined in detail the polymorphic and mutational status of the *KLK10* gene, using DNA isolated from normal tissues and from cancers of the breast, ovary, prostate, and testis. Bharaj *et al.* confirmed that the *KLK10* gene does not seem to be a target for somatic mutations in either breast, ovarian, prostate, or testicular cancer. A single nucleotide variation at codon 50, however, was associated

with increased prostate cancer risk. Recently, Li *et al.* demonstrated an important role for CpG island methylation in the loss of *KLK10* gene expression in breast cancer [142].

Despite the discovery of *KLK10* as a tumor suppressor through gene downregulation, recent data indicate that one major mechanism of *KLK10* inactivation may be at the epigenetic level. The frequent loss of *KLK10* expression indicates that inactivating the function of *KLK10* may be a critical step toward carcinogenesis. By treating *KLK10* nonexpressing cells with a demethylating agent and using methylation-specific PCR and sequence analysis of sodium bisulfite-treated genomic DNA, Li *et al.* [142] demonstrated a strong correlation between *KLK10* exon 3 hypermethylation and loss of *KLK10* mRNA expression in a panel of breast cancer cell lines and primary breast tumors. Furthermore, this study supports the notion that *KLK10* expression and its methylation status can be used as a molecular marker in breast cancer. These results justify using larger follow-up studies to evaluate the methylation status of *KLK10* as a screening tool for the detection of breast cancer, as well as other malignancies such as leukemia [143].

For the remaining human kallikrein genes, in none of them has methylation been tested as a potential mechanism for inactivation in the development or progression of cancer. Accumulating data provide indirect evidence that these kallikrein genes may be involved in the development of various malignancies, as a subset of these genes are downregulated in many cancers [43]. It is possible that some of these genes are epigenetically suppressed or silenced through CpG island methylation. Clearly, there is a need to characterize the CpG islands within these genes and to better understand the mechanism and role that epigenetic regulation plays within the human kallikrein locus.

## 10. Hormonal Regulation of Kallikreins

Steroid hormones, acting through their receptors, play important roles in the normal development and function of many organs. In addition, they are involved in the pathogenesis of many types of cancer [146]. Several reports confirmed that many kallikreins are under steroid hormone regulation in endocrine-related tissues and cell lines [100, 147–155].

An interesting observation is the tissue-specific pattern of hormonal regulation of several of these genes in different tissues. For example, *KLK4* is upregulated by androgen in prostate and breast cancer cell lines [148] and by estrogen in endometrial cancer cell lines [147]. Also, *KLK12* was found to be upregulated by androgens and progestins in prostate cancer cell lines and by estrogens and progestins in breast cancer cell lines [156]. *KLK14* and *KLK15* are mainly regulated by androgens. Recent preliminary kinetic and blocking

experiments indicate that this upregulation is mediated through the androgen receptor [155, 157].

In general, it can be concluded that most, if not all, kallikrein genes are regulated by steroid hormones, either predominantly by androgens or by estrogens/progestins/glucocorticoids. Because most of the data have been generated in cell lines, which contain variable amounts of various steroid hormone receptors, it will be very interesting to delineate the hormonal regulation of these genes *in vivo*. Manipulation of kallikrein gene expression by steroids may have therapeutic potential in some diseases such as cancer, psoriasis, or others.

Recently, Palmer *et al.*, working with human colon cancer cell lines, reported dramatic up-regulation of kallikreins 6 and 10 by  $1\alpha, 25$ -dihydroxyvitamin D3 [158]. This finding raises the possibility that some kallikreins could be regulated by a multitude of nuclear receptors.

## 11. Evolution of Kallikreins

The glandular kallikreins are simple secreted serine proteases. Some studies have investigated the phylogenetic relationship between different serine proteases, but no definitive conclusions regarding the glandular kallikreins could be drawn [62, 63, 159]. A more thorough phylogenetic analysis of the serine proteases is needed to elucidate the origin of the glandular kallikrein family.

A comparison of the genes in the glandular kallikrein family of man and mouse, combining phylogenetic analysis with a structural analysis of the loci, provided important clues about the evolution of the genes within this family [10]. The kallikreins from *KLK4* through to *KLK15* are well conserved in the two species, but the classical glandular kallikreins (*KLK1*, *KLK2*, and *KLK3*) differ significantly, and only *KLK1* (encoding tissue kallikrein) is present in both species. When the separation of the murine lineages from mammals of higher orders took place approximately 115 million years ago [160], 14 glandular kallikrein genes existed. These genes were *KLK4* to *KLK15* and two classical glandular kallikreins, *KLK1* and a progenitor of the human genes encoding PSA and hK2. After the division of the two species, this progenitor was silenced in the mouse lineage (this pseudogene is referred to as  $\Psi mGK$ ) and in the lineage leading to humans it was duplicated, resulting in the genes encoding PSA and hK2. *KLK1* was kept unaltered in human, but in mouse, it was extensively duplicated, resulting in another 24 paralogues. When studying the evolution of glandular kallikreins, it is tempting to speculate that the evolutionarily older and conserved kallikreins from *KLK4* to *KLK15* may be involved in processes that are more fundamental

than the younger classical glandular kallikreins. Further, the classical glandular kallikreins, which vary dramatically between species, appear to be involved in physiological processes that are more species specific.

## **12. Cross-Talk Between Kallikreins: A Possible Novel Enzymatic Cascade Pathway**

Interactions between serine proteases are common, and substrates of serine proteases are usually other serine proteases that are activated from an inactive precursor [66]. The involvement of serine proteases in cascade pathways is well documented. One important example is the blood coagulation cascade. Blood clots are formed by a series of zymogen activations. In this enzymatic cascade, the activated form of one factor catalyzes the activation of the next factor. Very small amounts of the initial factors are sufficient to trigger the cascade because of the catalytic nature of the process. These numerous steps yield a large amplification, thus ensuring a rapid and amplified response to trauma. A similar mechanism is involved in the dissolution of blood clots. A third important example of the coordinated action of serine proteases is the intestinal digestive enzymes. The apoptosis pathway is another important example of coordinated action of other types of proteases.

The cross-talk between kallikreins and the hypothesis that they are involved in a cascade enzymatic pathway are supported by strong, but mostly circumstantial evidence, as follows: many kallikreins are coexpressed in the same tissue (e.g., the adjacently localized kallikrein genes *KLK2*, *KLK3*, *KLK4*, and *KLK5* are all highly expressed in the prostate); some kallikreins have the ability to activate each other and the ability of other serine proteases to activate kallikreins (see following); the common patterns of steroid hormonal regulation; the parallel pattern of differential expression of many kallikreins in different malignancies (e.g., at least seven kallikrein genes are up-regulated in ovarian cancer, and at least seven kallikreins are down-regulated in breast cancer); and serine proteases commonly use other serine proteases as substrates.

Recent experiments have shown that hK3 can be activated by hK15 [161]. hK4 has also recently been shown to activate hK3 and does so much more efficiently compared to hK2 [106]. hK5 is predicted to be able to activate hK7 in the skin [103]. The activation of hK3 by hK2 is also possible. Although Takayama *et al.*, reported the ability of hK2 to activate hK3 [162], Denmeade *et al.* reported the opposite [105] and hypothesized that additional proteases may be required. It will be interesting to study all possible combinations of interactions among kallikreins, especially those

with expression in the same tissues. Bhoola *et al.* have recently provided strong evidence of the involvement of a “kallikrein cascade” in initiating and maintaining systemic inflammatory responses and immune-modulated disorders [163].

Kallikreins might also be involved in cascade reactions involving nonkallikrein substrates. This is evident from the reported, but questionable, ability of hK3 to digest insulin-like growth factor-binding protein [164] and to inactivate parathyroid hormone-related protein [165]. Similar properties were reported for rodent kallikreins. There is also experimental evidence that hK2 and hK4 can activate the pro-form of another serine protease, the urokinase-type plasminogen activator [106, 125]. As mentioned above, other serine proteases, such as enterokinase and trypsin, are predicted to be able to activate many kallikreins. Furthermore, hK4 can degrade prostatic acid phosphatase in seminal plasma; hK7 can degrade the alpha chain of native human fibrinogen, and it is hypothesized that it is involved in an apoptotic-like mechanism that leads to skin desquamation [166]. A proposed model for the involvement of kallikreins in a cascade-like reaction and its association to the pathogenesis of ovarian cancer has recently been published [167].

### **13. Isoforms and Splice Variants of the Human Kallikreins**

The mechanism of a single gene giving rise to greater than one mRNA transcript is referred to as differential splicing. This system is often tightly regulated in a cell-type or developmental stage-specific manner and increases genome complexity by generating different proteins from the same mRNA.

The presence of more than one mRNA form for the same gene is common among kallikreins. These variant mRNAs may result from alternative splicing, a retained intronic segment, or use of an alternative transcription initiation site. To date, there are at least 49 documented splice variants of the 15 kallikrein genes (Table 4), and more are currently being investigated (our unpublished data). Some of these variants may hold significant clinical value. Slawin *et al.* reported the prognostic significance of a splice-variant-specific RT-PCR assay for *KLK2* in detecting prostate cancer metastasis [168]. Nakamura *et al.* reported differential expression of the brain and prostate-types of *KLK11* among benign, hyperplastic, and malignant prostate cancer cell lines [169]. A novel ovarian cancer-specific variant of hK5 has been recently reported [170], and another *KLK5* transcript with a short 5'-untranslated region and a novel *KLK7* transcript with a long 3'-untranslated region were highly expressed in the ovarian cancer cell lines OVCAR-3 and PEO1, respectively, but were expressed at very low levels in normal ovarian

epithelial cells. Both Western blot and immunohistochemistry analyses have shown that these two enzymes are secreted from ovarian carcinoma cells. Thus, the short *KLK5* and long *KLK7* transcripts may be useful as tumor markers for epithelial-derived serous carcinomas [170].

Some of these alternatively spliced forms were also found to be tissue specific. A 1.5-kb transcript of *KLK14* was only found in the prostate, and another 1.9 kb transcript was found only in skeletal muscle [171]. Several splice variants of *KLK13* were found to be testis specific [172]. Type 2 neuropeptide (NPY) is preferentially expressed in the hippocampus of the human adult brain [173], and a new splice variant of *KLK4* was isolated from prostatic tissue [174]. A *KLK6* splice variant (GenBank accession no. AY279381) was strongly expressed in adult brain compared to fetal brain. Some of these splice variants were found to be translated [175, 176].

Evolutionarily conserved sequences ensure that the 3' and 5' splice sites are correctly cleaved and the two ends properly joined. These consensus sequences contain invariant dinucleotides at each end—GT (donor site) and AG (acceptor site)—and are associated with a more flexible sequence, AG:GT(A/G)AGT...CAG:G. However, exceptions to the tightly regulated splice sites can arise. An alternative GT-GC intron may exist, but unlike a possible AT-AC intron boundary, it will still be processed by the same splicing pathway as the conventional GT-AG introns. The GT-GC boundary is present in some kallikrein splice variants including the 5'-untranslated region of *KLK5* splice variant 2 (GenBank accession no. AY279381) and intron 3 for *KLK10* transcript variant 2 (GenBank accession no. 145888).

## 14. Kallikreins in Normal Physiology

Little is known about the physiological functions of kallikreins in normal tissues. However, accumulating evidence indicates that kallikreins might have diverse functions, depending on the tissue and circumstances of expression. hK1 exerts its biological activity mainly through the release of lysyl-bradykinin (kallidin). It cleaves low-molecular weight kininogen to produce vasoactive kinin peptides. Intact kinin binds to bradykinin B<sub>2</sub> receptor in target tissues and exerts a broad spectrum of biological effects including blood pressure reduction via vasodilatation, smooth muscle relaxation or contraction, pain induction, and mediation of the inflammatory response [177]. Low renal synthesis and urinary excretion of tissue kallikrein have been repeatedly linked to hypertension in animals and humans [178]. It has also been reported that tissue kallikrein cleaves kininogen substrate to produce vasoactive kinin peptides that have been implicated in the proliferation of vascular smooth muscle cells. Abnormalities of the

TABLE 4  
 REPORTED SPLICE VARIANTS OF THE HUMAN KALLIKREIN GENES

Gene and splice variant name	Splice variant description	Reference
<i>KLK1</i>		
Intron containing kallikrein mRNA	Deletion of exon 2 + alternative first exon	[257]
ND <sup>a</sup>	104 bp deletion at beginning of exon 5	[258]
<i>KLK2</i>		
GK-10A	Additional 37 bp at the end of exon 4 + early 3' UTR <sup>b</sup>	[150]
AF188745*	13-bp deletion at beginning of exon 4 + 37-bp extension at end of exon 4 + deletion of exon 5 + early 3' UTR <sup>b</sup>	[259]
AF188747*	37-bp extension at end of exon 4 + early 3' UTR <sup>b</sup>	[259]
AF336106*	Extension of exon 1 + deletion of exons 2, 3, 4, and 5 + early 3' UTR <sup>b</sup>	[1]
<i>KLK3</i>		
PSA-RP1/PA 525	Alternative acceptor site for exon 5 starts 442 bp upstream of classical <sup>c</sup> exon 5 → 2 variations of PSA-RP1 with different 3' UTRs <sup>b</sup>	[175]
PSA-RP2	Extension from exon 3-4 + deletion of coding exon 4 and 5 + early 3' UTR <sup>b</sup>	[2]
PSA-RP3	129-bp deletion in beginning of exon 3	[176]
PSA-RP4	123-bp deletion in middle of exon 3	[2]
PSA-RP5	386-bp extension at end of exon 4 + deletion of coding exon 5 + early 3' UTR <sup>b</sup>	[175]
PSA-LM	269-bp extension of coding region at end of exon 1 + early 3' UTR <sup>b</sup> → 2 variations of PSA-LM with different 3' UTRs <sup>b</sup>	[1,2]
PA 424	Joining of exons 3 and 4 + 105-bp deletion at end of exon 4 + deletion of exon 5 + early 3' UTR <sup>b</sup>	[45, 260]
<i>KLK4</i>		
ND <sup>a</sup>	12-bp extension at end of exon 2 + early 3' UTR <sup>b</sup>	[174, 261]
KLK4-S	Deletion of exon 1 and 4 + exon 2 (alternative first exon) starts 61 bp downstream beginning of classical <sup>c</sup> exon 2 + early 3' UTR <sup>b</sup>	[147]
ND <sup>a</sup>	Additional sequence in 5' UTR <sup>b</sup>	[41]



KLK4-L	Exon 3 extended to exon 4 + exon 2 (alternative first exon) starts 61 bp downstream beginning of classical <sup>c</sup> exon 2 + early 3' UTR <sup>b</sup>	[261]
ND <sup>a</sup>	Alternative first exon starts 61 bp downstream beginning of classical <sup>c</sup> exon 2	[261]
KLK5		
Kallikrein 5 splice variant 1	203-bp deletion at end of noncoding exon 1 (5' UTR <sup>b</sup> ) + 45 bp upstream extension in beginning of noncoding exon 1	AY279380*
Kallikrein 5 splice variant 2	135-bp deletion in middle of noncoding exon 1 (5' UTR <sup>b</sup> ) + 45 bp upstream extension in beginning of noncoding exon 1	AY279381*
Ovarian cancer klk5-long	40 bp upstream extension at beginning of noncoding exon 1	[170]
Ovarian cancer klk5-short	40 bp upstream extension at beginning of noncoding exon 1 + 204-bp deletion at end of noncoding exon 1 (5' UTR <sup>b</sup> )	[170]
KLK6		
Kallikrein 6 splice variant 1	Deletion of coding exon 2 + early 3' UTR <sup>b</sup>	AY279383*
KLK7		
KLK7 long	144 bp upstream extension to noncoding exon 1	[170]
KLK7 transcript variant 2	Noncoding exon 1 starts 36 bp downstream from the end of classical <sup>c</sup> noncoding exon 1	[152]
KLK8		
Neuropsin type 2	135 bp upstream extension of second coding exon	[173]
Neuropsin type 3	Deletion of coding exons 2 and 3	[233]
Neuropsin type 4	Deletion of exons 2, 3, and 4 + early 3' UTR <sup>b</sup>	[233]
TADG14	Additional 491 bp in 5' UTR <sup>b</sup>	[262]
KLK9		
KLK9 splice variant-1	106-bp deletion at the end of exon 3 + late 3' UTR <sup>b</sup>	AF135026
KLK10		
KLK10 transcript variant 2	Noncoding exon 1 starts 122 bp downstream end of classical <sup>c</sup> noncoding exon 1 + second noncoding exon starts 3 bp downstream beginning of classical <sup>c</sup>	[239]
KLK11		
Brain-type TLSP	Prostate-type variant is located 282 bp downstream end of brain-type specific noncoding exon 1	[263]
KLK12		
KLK12 related protein-1	Split last coding exon with an intervening intron of 129 bp + late 3' UTR <sup>b</sup>	[156]
KLK12 related protein-2	Deletion of coding exon 3 + early 3' UTR <sup>b</sup>	[156]

(continues)

TABLE 4 (Continued)

Gene and splice variant name	Splice variant description	Reference
<i>KLK13</i>		
Short KLK-L4 variant	211-bp deletion at end of coding exon 3 + early 3' UTR <sup>b</sup>	[90]
Long form	Additional exon between exons 1 and 2 + early 3' UTR <sup>b</sup>	[172]
L4E-S	Alternative first exon + 211-bp deletion at end of exon + early 3' UTR <sup>b</sup>	[172]
L4E-L	Alternative first exon + 80-bp extension at end of exon 3	[172]
L4D-S	Additional exon between exons 1 and 2 + 211-bp deletion at end of exon 3 + early 3' UTR <sup>b</sup>	[172]
L4D-L	Additional exon between exons 1 and 2 + 80-bp extension at end of coding exon 3 + early 3' UTR <sup>b</sup>	[172]
Kallikrein 13 splicing variant 2	211-bp deletion at end of coding exon 3	[90]
Kallikrein 13 splicing variant 3	Deletion of coding exons 2 and 3	[90]
<i>KLK14</i>		
ND <sup>a</sup>	1.5-kb transcript (not characterized)	[171]
ND <sup>a</sup>	1.9-kb transcript (not characterized)	[171]
<i>KLK15</i>		
KLK15 splice variant 1	118-bp deletion at end of exon 3 + early 3' UTR <sup>b</sup>	[51]
KLK15 splice variant 2	Deletion of exon 4 + early 3' UTR <sup>b</sup>	[51]
KLK15 splice variant 3	118 bp deletion at end of exon 3 + deletion of exon 4	

\* Accession numbers in GenBank (variant name may not be determined, or submission may be unpublished).

<sup>a</sup>ND: not defined.

<sup>b</sup>Untranslated region (noncoding).

<sup>c</sup>“Classical” refers to the published report of the gene that follows the definition of a kallikrein gene, as described in the text.

tissue kallikrein–kinin system have been implicated in the pathogenesis of hypertension and cardiovascular and renal disorders [179].

An hK1 knockout mouse has recently been generated and found to be unable to generate significant levels of kinins in most tissues and develop cardiovascular abnormalities early in adulthood despite normal blood pressure [178].

However, the diverse expression pattern of hK1 has led to the suggestion that the functional role of this enzyme may be specific to different cell types [177]. Apart from its kininogenase activity, tissue kallikrein has been implicated in the processing of growth factors and peptide hormones in light of its presence in pituitary, pancreas, and other tissues. As summarized by Bhoola *et al.* [177], hK1 has been shown to cleave proinsulin, low-density lipoprotein, prorenin, angiotensinogen, vasoactive intestinal peptide, procollagenase, and the precursor of atrial natriuretic factor.

Seminal plasma hK2 was found to be able to cleave seminogelin I and seminogelin II, but at different cleavage sites and with lower efficiency than hK3 [180]. Because the amount of hK2 in seminal plasma is much lower than hK3 (1%–5%), the contribution of hK2 in the process of seminal clot liquefaction is expected to be relatively small [31].

Because hK3 is present at very high levels in seminal plasma, most studies focused on its biological activity within this fluid. Lilja has shown that hK3 rapidly hydrolyzes seminogelin I and seminogelin II, as well as fibronectin, resulting in liquefaction of the seminal clot after ejaculation [181]. Several other potential substrates for hK3 have been identified, including insulin-like growth factor–binding protein 3, TGF- $\beta$ , parathyroid hormone-related peptide, and plasminogen [182]. The physiological relevance of these findings is still not clear.

The mouse and porcine orthologues of hK4 were originally designated enamel matrix serine protease because of their predicted role in normal teeth development [41]. The human *KLK4* gene, however, was shown to be highly expressed in the prostate, pointing to the possibility that it has a different function in humans. hK7 and, more recently, hK5 were found to be highly expressed in the skin, and it is believed that they are involved in skin keratinization and desquamation [183]. hK6, hK8, and hK11 are highly expressed in the central nervous system, where they are thought to play a role in neural plasticity.

Another possible mechanism for kallikrein action in physiology and pathobiology is the activation of proteinase-activated receptors (PARs). PAR is a recently described family of G-protein-coupled receptors with seven transmembrane domains that are stimulated by cleavage of their N-termini by a serine protease rather than by ligand-receptor interaction [184–186]. Four PARs have been identified so far, of which PAR1, PAR3,

and PAR4 are activated by thrombin, whereas PAR2 is activated by trypsin or mast cell tryptase. Activation of these receptors elicits different responses in several tissues. In addition, they switch-on cell signaling pathways (e.g., the MAP-kinase pathway) leading to cell growth and division. Because most kallikreins have trypsin-like activity, they might also be involved in such mechanisms. The possible cleavage of PARs by kallikreins is an exciting new avenue of investigation, but no published data exist on this issue.

## 15. Association of Kallikreins with Human Diseases

Some kallikrein genes have been associated with the pathogenesis of human diseases. The *KLK1* gene is involved in inflammation [8], hypertension [187], renal nephritis, and diabetic renal disease [188]. The relationships between hK5 and hK7 and skin diseases, including pathological keratinization and psoriasis, have already been reported [189, 190]. Much research is now focusing on the relation of kallikreins to diseases of the central nervous system (CNS) and skin, as well as to malignancy, as discussed below.

### 15.1. KALLIKREINS IN CNS DISEASES

Many kallikreins seem to play important physiological roles in the CNS. In mouse, neuropsin appears to have an important role in neural plasticity, and the amount of neuropsin mRNA is related to memory retention after a chemically induced ischemic insult [191]. The human neuropsin gene (hK8) was first isolated from the hippocampus. Recent reports describe the association of hK8 expression with diseases of the CNS, including epilepsy [192, 193]. In addition, an 11.5-fold increase in *KLK8* mRNA levels in Alzheimer's disease (AD) hippocampus compared to controls was recently reported [194]. The same study showed that *KLK1*, *KLK4*, *KLK5*, *KLK6*, *KLK7*, *KLK8*, *KLK10*, *KLK11*, *KLK13*, and *KLK14* are expressed in both the cerebral cortex and hippocampus, whereas *KLK9* is expressed in the cortex but not the hippocampus [194]. Another kallikrein, *KLK6*, was shown to have amyloidogenic activity in the AD brain [107, 195], indicating that it may play a role in AD development (107). *KLK6* was also found to be localized in perivascular cells and microglial cells in human AD brain [107]. Scarisbrick *et al.* have shown that hK6 is abundantly expressed by inflammatory cells at sites of CNS inflammation and demyelination in animal models of multiple sclerosis and in human lesions at autopsy, prompting the researchers to postulate that hK6 in inflammatory CNS lesions may promote demyelination [196, 197]. *KLK11*, another newly discovered kallikrein, was isolated from brain hippocampus cDNA and is thought to play a role in brain plasticity [198]. Similarly, *KLK5* and *KLK14* are also expressed at high levels

in the brain [153, 199] and might have roles in normal and aberrant brain physiology. A recent report showed significant alterations of hK6, hK7, and hK10 concentrations in CSF of patients with AD and frontotemporal dementia [200]. For a more detailed discussion about the role of kallikreins in the CNS, we refer the reader to our recent review [201].

## 15.2. KALLIKREINS IN SKIN DISEASES

The epidermis forms the external surface of the skin and is composed of differentiated keratinocytes that form four layers: the stratum basale, the stratum spinosum, the stratum granulosum, and the stratum corneum, where keratinocytes have been transformed into corneocytes. The stratum corneum functions as the protective, virtually water-impermeable skin barrier against external insults including desiccation and the entry of noxious chemicals and microbes. To maintain this barrier, old corneocytes are continuously desquamated from the stratum corneum by both stratum corneum trypsin-like and stratum corneum chymotrypsin-like enzymes [202]. The expression of hK5 and hK7 [203] and of several kallikrein mRNAs [204] in the upper epidermis (the stratum granulosum or the stratum corneum), indicate that kallikreins may be the stratum corneum serine proteases responsible for the desquamation of corneocytes [205]. A quantitative ELISA assay showed high concentrations of hK7 [206] and hK8 [78] in skin tissue extracts. In addition, given that kallikrein proteins or mRNAs are also expressed in skin appendages such as sweat glands, sebaceous glands, and hair follicles [203, 205, 207], kallikrein activities may be related to the maturation and secretion of sebum and sweat and to hair growth [205].

In addition, kallikrein expression may also be involved in the pathogenesis of several skin diseases. In psoriasis, an inflammatory skin disease, various kallikrein mRNAs were shown to be up-regulated in the upper epidermis [204] and associated with the conversion of the inactive hK7 precursor to active hK7 in the psoriatic lesion [189]. In ichthyoses and squamoproliferative disorders, hK7 expression was found to be low [208]. Transgenic mice overexpressing hK7 showed increased epidermal thickness, hyperkeratosis, and a dermal inflammation with pruritus [209] and expression of MHC II antigen [210]. These data indicated that hK7 may lead to skin changes that contribute to development of inflammatory skin diseases [210]. hK8-deficient mice showed a prolonged recovery of ultraviolet B-irradiated skin, indicating that hK8 might be involved in the process of differentiation [211]. Psoriasis vulgaris, seborrheic keratosis, lichen planus, and squamous cell carcinoma also display a high density of *KLK8* mRNA [207].

In Netherton syndrome, a severe inherited skin disorder, the molecular defect is thought to involve mutations of the serine protease inhibitor

TABLE 5  
 PROGNOSTIC VALUE OF KALLIKREIN GENES/PROTEINS IN OVARIAN CANCER

Kallikrein gene/protein and sample used	Method	Clinical applications	Reference
<i>KLK4</i> mRNA from normal and cancerous ovarian tissues	RT-PCR <sup>a</sup>	<p><i>Unfavorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Overexpressed in patients with late-stage disease, higher-grade tumors, and no response to chemotherapy</li> <li>• Associated with shorter DFS<sup>b</sup> and OS<sup>c</sup></li> <li>• Independent indicator of poor prognosis in patients with low-grade tumors</li> </ul>	[226]
mRNA from normal, benign, and cancerous ovarian tissues and late-stage serous ovarian cancer cell lines	SQ-RT-PCR, <sup>d</sup> Southern blot	<p><i>Unfavorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Overexpressed in late-stage serous ovarian tumor tissues and cell lines</li> </ul>	[264]
<i>KLK5</i> mRNA from normal and cancerous ovarian tissues	RT-PCR	<p><i>Unfavorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Overexpressed in patients with late-stage disease and higher-grade tumors</li> <li>• Associated with shorter DFS and OS</li> <li>• Independent indicator of poor prognosis in patients with low-grade tumors</li> </ul>	[227]
<i>KLK5/hK5</i> mRNA and extracts from normal, benign and cancerous ovarian tissues and late-stage serous ovarian cancer cell lines	SQ-RT-PCR, Southern, Northern, and Western blots and immunohistochemistry	<p><i>Unfavorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Overexpressed in ovarian tumor tissues and cell lines mainly of late stage and serous histotype</li> </ul>	[170]

hK5	Ovarian cancer cytosols	Immunoassay	<p><i>Unfavorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Overexpressed in patients with late stage disease and higher grade tumors</li> <li>• Associated with shorter DFS and OS</li> <li>• Independent indicator of poor prognosis in patients with high-grade tumors and optimal debulking success</li> </ul>	Our unpublished data
KLK6/hK6	mRNA and extracts from normal, benign, and cancerous ovarian tissues	SQ-RT-PCR, Northern blot, immunohistochemistry	<p><i>Unfavorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Overexpressed in ovarian cancer tissues</li> </ul>	[228]
hK6	Ovarian cancer cytosols	Immunoassay	<p><i>Unfavorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Overexpressed in patients with late-stage disease and serous tumors</li> <li>• Associated with shorter DFS and OS</li> <li>• Independent indicator of poor prognosis in patients with low-grade tumors and optimal debulking success</li> </ul>	[229]
	Serum from normal women, women with benign disease and ovarian cancer	Immunoassay	<p><i>Unfavorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Serum hK6 levels elevated in cancer vs. normal</li> <li>• Higher serum hK6 I patients with late-stage disease, higher grade, serous tumors, suboptimal debulking, and a poor response to chemotherapy</li> <li>• Indicator of decreased DFS and OS</li> </ul>	[217]
KLK7	mRNA and extracts from normal and cancerous ovarian tissues	SQ-RT-PCR, Northern and Western blots and immunohistochemistry	<p><i>Unfavorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Overexpressed in ovarian cancer tissues</li> </ul>	[265]

(continues)

TABLE 5 (Continued)

Kallikrein gene/protein and sample used	Method	Clinical applications	Reference
<p><i>KLK7/hK7</i> mRNA from normal, benign, and cancerous ovarian tissues and late-stage serous ovarian cancer cell lines</p>	<p>SQ-RT-PCR, Southern, Northern, and Western blots and immunohistochemistry</p>	<p><i>Unfavorable</i> prognosis:  <ul style="list-style-type: none"> <li>• Overexpressed in ovarian tumor tissues and cell lines mainly of late stage and serous histotype</li> </ul> </p>	[170]
<p>mRNA from cancerous ovarian tissue</p>	Q-RT-PCR <sup>e</sup>	<p><i>Unfavorable</i> prognosis:  <ul style="list-style-type: none"> <li>• Overexpressed in higher-grade tumors</li> <li>• Associated with shorter DFS</li> <li>• Independent indicator of poor prognosis in patients with low-grade tumors and optimal debulking success</li> </ul> </p>	[230]
<p><i>KLK8</i> mRNA from ovarian cancer tissues</p>	RT-PCR	<p><i>Favorable</i> prognosis:  <ul style="list-style-type: none"> <li>• Overexpressed in lower-grade tumors</li> <li>• Associated with longer DFS and OS</li> <li>• Independent indicator of longer DFS</li> </ul> </p>	[233]
<p><i>KLK9</i> mRNA from ovarian cancer tissues</p>	Q-RT-PCR	<p><i>Favorable</i> prognosis:  <ul style="list-style-type: none"> <li>• Overexpressed in patients with early-stage disease and optimal debulking success</li> <li>• Associated with longer DFS and OS</li> <li>• Independent indicator of prolonged DFS in patients with low-grade tumors and optimal debulking success</li> </ul> </p>	[234]



hK10	Normal, benign, and cancerous ovarian cytosols	Immunoassay	<p data-bbox="228 772 261 1073"><i>Unfavorable</i> prognosis: [91]</p> <ul data-bbox="269 407 451 1073" style="list-style-type: none"> <li>• Overexpressed in cancer patients with late-stage disease, serous tumors, and suboptimal debulking success</li> <li>• Associated with shorter DFS and OS</li> <li>• Independent indicator of DFS and OS in patients with late-stage tumors</li> </ul>
Serum from normal women, women with benign disease and ovarian cancer	Immunoassay	<p data-bbox="488 772 521 1073"><i>Unfavorable</i> prognosis: [218]</p> <ul data-bbox="529 407 753 1073" style="list-style-type: none"> <li>• Serum hK10 levels elevated in cancer vs. normal</li> <li>• Higher serum hK10 in patients with late-stage disease, higher-grade, serous tumors, suboptimal debulking, and a poor response to chemotherapy</li> <li>• Indicator of decreased DFS and OS</li> <li>• Independent indicator of OS</li> </ul>	
hK11	Ovarian cancer cytosols	Immunoassay	<p data-bbox="794 806 826 1073"><i>Favorable</i> prognosis: [235]</p> <ul data-bbox="834 407 1089 1073" style="list-style-type: none"> <li>• Overexpressed in patients with early-stage disease, pre-/perimenopausal status, and who responded to chemotherapy</li> <li>• Associated with longer DFS and OS</li> <li>• Independent indicator of OS</li> <li>• Independent indicator of DFS and OS in patients with low-grade tumors</li> </ul>
hK13	Ovarian cancer cytosols	Immunoassay	<p data-bbox="1130 806 1162 1073"><i>Favorable</i> prognosis: [266]</p> <ul data-bbox="1170 407 1318 1073" style="list-style-type: none"> <li>• Overexpressed in early-stage disease, and patients with no residual tumor after surgery and optimal debulking success</li> <li>• Independent indicator of longer DFS and OS</li> </ul>

(continues)

TABLE 5 (Continued)

Kallikrein gene/protein and sample used	Method	Clinical applications	Reference
<p><i>KLK14</i> mRNA from normal, benign and cancerous ovarian tissues</p>	Q-RT-PCR	<p><i>Favorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Stepwise decrease in the amount <i>KLK14</i> mRNA (normal &gt; benign &gt; cancerous tissues)</li> <li>• Overexpressed in patients with early-stage disease and optimal debulking success, who responded to chemotherapy</li> <li>• Independent indicator of longer DFS and OS</li> </ul>	[157]
<p>Serum and tissue from ovarian cancer patients</p>	ELISA	<p>Diagnosis:</p> <ul style="list-style-type: none"> <li>• Elevated serum levels in 65% of ovarian cancer patients vs. normal</li> <li>• Higher levels in 40% of ovarian cancer tissues compared to normal</li> </ul>	[258]
<p><i>KLK15</i> mRNA from benign and cancerous ovarian tissues</p>	Q-RT-PCR	<p><i>Unfavorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Higher levels in cancerous tissues</li> <li>• Independent indicator of decreased DFS and OS</li> </ul>	[232]

<sup>a</sup> Reverse transcriptase-polymerase chain reaction.

<sup>b</sup> Disease-free survival.

<sup>c</sup> Overall survival.

<sup>d</sup> Semicquantitative reverse transcriptase polymerase chain reaction (RT-PCR).

<sup>e</sup> Quantitative RT-PCR.

Kazal-type 5 (SPINK5), also known as LEKT1 [212–214]. It is likely that inactivation of the inhibitor leads to overactivity of hK5, hK7, and possibly other kallikreins in skin. The same general mechanism may apply to psoriasis (our unpublished data). Clearly, a possible kallikrein cascade in skin, including active enzymes and inhibitors, needs to be further investigated.

## 16. Kallikreins and Cancer

### 16.1. OVERVIEW

The association of kallikreins with cancer is well established. PSA (hK3) and, more recently, human glandular kallikrein (hK2) are useful biomarkers for prostate cancer. A more detailed discussion about hK2 and hK3 as cancer biomarkers can be found elsewhere [31]. In addition to its established role in prostate cancer diagnosis and monitoring, recent reports indicate that hK3 can be useful as a marker for breast cancer prognosis [215].

With the identification and characterization of all members of the kallikrein gene family, accumulating evidence indicates that other kallikreins might be also related to hormonal (e.g., breast, prostate, testicular, and ovarian cancers) and other malignancies. *KLK6* and *KLK10* were originally isolated by differential display from breast cancer libraries [216].

At the protein level, recent reports demonstrate that kallikrein proteins can be useful serum biomarkers for diagnosis and prognosis of cancer. In addition to hK3 and hK2, hK6 and hK10 are emerging diagnostic markers for ovarian cancer [217, 218]. Also, hK11 was shown to be a potential marker for ovarian and prostate cancer [219]. A synthetic hK1 inhibitor was recently found to suppress cancer cell invasiveness in human breast cancer cell lines [220]. hK1 was immunolocalized in the giant cells of squamous cell carcinoma of the esophagus and gastric carcinoma [163].

In Tables 5–8, we summarize published data on the analysis of kallikrein genes and proteins in tumor tissue extracts and serum of cancer patients for the purpose of disease diagnosis, monitoring, and prognosis. As discussed below, some kallikreins are very promising new cancer biomarkers.

The differential regulation of certain kallikreins in more than one type of cancer has been repeatedly reported. The phenotype of cells and tissues, benign or malignant, ultimately depends on which proteins, and at what level, are expressed at any time. Ultimately, the pathological classification of human neoplasia, which is now based on histological features, will be replaced with a biological portrait of different tumors, including hundreds or thousands of differentially expressed genes [221].

TABLE 6  
 PROGNOSTIC/PREDICTIVE VALUE OF KALLIKREIN GENES/PROTEINS IN BREAST CANCER

Kallikrein gene/protein and sample used	Method	Clinical applications	Reference
hK3/PSA Breast cancer cytosols	Immunoassay	<p><i>Favorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Overexpressed in younger patients with early-stage disease, small ER<sup>-</sup>-positive, low S-phase, low-cellularity diploid tumors</li> <li>• Associated with a longer DFS<sup>b</sup> and OS<sup>c</sup></li> <li>• Independent indicator of increased DFS for all patients as well as node-positive, ER-negative</li> </ul> <p><i>Favorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Overexpressed in younger, pre-/perimenopausal patients with smaller, steroid hormone receptor-positive tumors</li> <li>• Associated with a longer OS</li> </ul> <p>Predictive value:</p> <ul style="list-style-type: none"> <li>• Higher hK3 levels associated with a poor response to tamoxifen therapy</li> </ul>	[267, 268]
Breast cancer cytosols	Immunoassay	<p><i>Favorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Overexpressed in younger, pre-/perimenopausal patients with smaller, steroid hormone receptor-positive tumors</li> <li>• Associated with a longer OS</li> </ul> <p>Predictive value:</p> <ul style="list-style-type: none"> <li>• Higher hK3 levels associated with a poor response to tamoxifen therapy</li> </ul>	[269]
<i>KLK5</i> mRNA from breast cancer tissues	Q-RT-PCR <sup>d</sup>	<p><i>Unfavorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Overexpressed in pre-/perimenopausal, node-positive patients with ER-negative tumors</li> <li>• Independently associated with decreased DFS and OS</li> <li>• Independent indicator of shorter DFS and OS in node-positive patients with large tumors</li> <li>• Associated with shorter DFS in patients with low-grade tumors</li> </ul>	[93]
<i>KLK9</i> mRNA from breast cancer tissues	Q-RT-PCR	<p><i>Favorable</i> prognosis:</p> <ul style="list-style-type: none"> <li>• Overexpressed in patients with early-stage disease and small tumors</li> <li>• Independently associated with increased DFS and OS</li> <li>• Independent indicator of prolonged DFS and OS in patients with ER and PR<sup>c</sup>-negative tumors</li> </ul>	[238]

hK10				[239]
Breast cancer cytosols	Immunoassay		<i>Predictive value:</i>	
			<ul style="list-style-type: none"> <li>Higher hK10 levels independently associated with a poor response to tamoxifen therapy</li> </ul>	
<i>KLK13</i>				[89]
mRNA from breast cancer tissues	Q-RT-PCR		<i>Favorable prognosis:</i>	
			<ul style="list-style-type: none"> <li>Overexpressed in older, estrogen receptor–positive patients</li> <li>Associated with a prolonged DFS and OS</li> <li>Independent indicator of longer DFS and OS in node-, ER-, and PR-positive patients with low-grade tumors</li> </ul>	
<i>KLK14</i>				[94]
mRNA from breast cancer tissues	Q-RT-PCR		<i>Unfavorable prognosis:</i>	
			<ul style="list-style-type: none"> <li>Overexpressed in patients with advanced-stage disease</li> <li>Independent indicator of shorter DFS<sup>b</sup> and OS<sup>c</sup></li> <li>Independent indicator of shorter DFS and OS in patients with a tumor size <math>\leq 2</math> cm and positive nodal, ER,<sup>d</sup> and PR<sup>e</sup> status</li> </ul>	
Serum from breast cancer patients	ELISA		<i>Diagnosis:</i>	[270]
			<ul style="list-style-type: none"> <li>Overexpressed in patients with advanced-stage disease</li> <li>Elevated serum hK14 levels in 40% breast cancer patients vs. normal</li> </ul>	
<i>KLK15</i>				[155]
mRNA from breast cancer tissues	Q-RT-PCR		<i>Favorable prognosis:</i>	
			<ul style="list-style-type: none"> <li>Overexpressed in node-negative patients</li> <li>Independently associated with a longer DFS and OS</li> <li>Independent indicator of longer DFS and OS in patients with lower-grade, ER- and PR-negative tumors</li> </ul>	

<sup>a</sup>Estrogen receptor.

<sup>b</sup>Disease-free survival.

<sup>c</sup>Overall survival.

<sup>d</sup>Quantitative reverse transcriptase polymerase chain reaction.

<sup>e</sup>Progesterone receptor.

TABLE 7  
 PROGNOSTIC VALUE OF KALLIKREIN GENES/PROTEINS IN PROSTATE CANCER

Kallikrein gene/protein and sample used	Method	Clinical applications	Reference
<i>KLK5</i> mRNA from matched normal and prostate cancer tissues	Q-RT-PCR <sup>a</sup>	<i>Favorable</i> prognosis: <ul style="list-style-type: none"> <li>• Down-regulated in cancer vs. normal prostate tissues</li> <li>• Higher levels associated with low grade tumors and low Gleason score</li> </ul>	[242]
<i>KLK11</i> mRNA from matched normal and prostate cancer tissues	Q-RT-PCR	<i>Favorable</i> prognosis: <ul style="list-style-type: none"> <li>• Overexpressed in cancer vs. normal prostate tissues</li> <li>• Prostate-specific splice variant associated with early-stage disease, lower tumor grade, and Gleason score</li> </ul>	[271]
<i>KLK14</i> mRNA from matched normal and prostate cancer tissues	Q-RT-PCR	<i>Unfavorable</i> prognosis: <ul style="list-style-type: none"> <li>• Overexpressed in cancer vs. normal prostate tissues</li> <li>• Overexpressed in patients with late-stage disease, high-grade tumors, and higher Gleason score</li> </ul>	[272]
<i>KLK15</i> mRNA from matched normal and prostate cancer tissues	Q-RT-PCR	<i>Unfavorable</i> prognosis: <ul style="list-style-type: none"> <li>• Overexpressed in cancer vs. normal prostate tissues</li> <li>• Overexpressed in patients with late-stage disease, high-grade tumors, and higher Gleason score</li> </ul>	[273]

<sup>a</sup>Quantitative reverse transcriptase polymerase chain reaction.

TABLE 8  
 PROGNOSTIC VALUE OF KALLIKREIN GENES/PROTEINS IN TESTICULAR CANCER

Kallikrein gene/protein and sample used	Method	Clinical applications	Reference
<i>KLK5</i> mRNA from matched normal and testicular cancer tissues	Q-RT-PCR <sup>a</sup>	<i>Favorable</i> prognosis: <ul style="list-style-type: none"> <li>• Down-regulated in cancer vs. normal testicular tissues</li> <li>• Overexpressed in smaller, early stage, nonseminomas</li> </ul>	[243]
<i>KLK10</i> mRNA from matched normal and testicular cancer tissues	RT-PCR	<ul style="list-style-type: none"> <li>• Down-regulated in cancer vs. normal testicular tissues</li> </ul>	[244]
<i>KLK13</i> mRNA from matched normal and testicular cancer tissues	RT-PCR	Testicular cancer-specific splice variants	[171]
<i>KLK14</i> mRNA from matched normal and malignant testicular tissues	RT-PCR	<i>Favorable</i> prognosis: <ul style="list-style-type: none"> <li>• Down-regulated in cancer vs. normal testicular tissues</li> </ul>	[199]

<sup>a</sup>Quantitative reverse transcriptase polymerase chain reaction (RT-PCR).

## 16.2. PROGNOSTIC AND PREDICTIVE VALUE OF KALLIKREINS IN HORMONE-DEPENDENT CANCERS

### 16.2.1. Ovarian Cancer

Epithelial ovarian cancer is the most lethal gynecologic malignancy [222]. The high mortality rate is usually ascribed to late diagnosis, as epithelial ovarian tumors commonly lack early warning symptoms. Furthermore, ovarian carcinomas often lack definite precursor lesions and are quite heterogeneous, and the molecular pathways underlying their progression are still elusive. Thus, many attempts have been made to predict the biology of ovarian tumors to determine prognosis and develop individualized treatment strategies. The International Federation of Gynecology and Obstetrics stage at diagnosis represents the major prognostic factor in ovarian cancer. International Federation of Gynecology and Obstetrics stage I patients have a 5-year survival of 80%–90%, compared with only 15%–20% for women with stage III and IV disease [223]. Other conventional prognostic markers include tumor grade, patient age, residual tumor after surgery, histology, and the presence or absence of ascites [224]. The potential diagnostic or prognostic role of kallikreins in ovarian cancer is summarized in Table 5.

Recently, an *in silico* analysis of kallikrein gene expression in ovarian cancer was performed by using the databases of the Human Genome Anatomy Project. This study showed that at least seven kallikreins are upregulated in ovarian cancer compared to normal ovarian tissues. These results were also confirmed at the protein level [126]. A recent review describing the prognostic value of 12 of 15 members of the human kallikrein family in ovarian cancer has also been recently published [225]. Other studies have shown that six kallikreins, namely, *KLK4*, *KLK5/hK5*, *KLK6/hK6*, *KLK7*, *hK10*, and *KLK15*, are markers of poor prognosis in ovarian cancer [226–232]. That is, higher kallikrein mRNA or protein levels were found to correlate with more aggressive forms of this disease and with a decreased disease-free and overall survival of patients. The remaining subset of kallikreins, namely, *KLK8*, *KLK9*, *hK11*, *hK13*, and *KLK14*, seem to be markers of favorable prognosis [157, 233–235] (and our unpublished data). 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 nonserous tumors. These data indicate that certain kallikreins may have prognostic



value in subgroups of patients stratified by histotype. A recent report demonstrated a higher expression of both *KLK5/hK5* and *KLK7/hK7* in ovarian carcinomas, especially late-stage serous carcinomas, compared with normal ovaries and benign adenomas [170]. Novel *KLK5* and *KLK7* splice variants such as the short *KLK5* and long *KLK7* transcripts may be useful as tumor markers for epithelial-derived serous carcinomas [170].

### 16.2.2. Breast Cancer

Breast cancer is the most prevalent malignancy among women worldwide, accounting for 21% of all female cancers and ranking third overall when both sexes are considered [236]. Although the increased use of screening by mammography for early disease diagnosis and the widespread administration of systemic adjuvant therapies have led to a slight decline in mortality rates, breast cancer is still the leading cause of death from cancer in women, causing over 39,800 deaths in the United States annually [222]. The optimal management of breast cancer patients involves a multidisciplinary approach, including the use of biomarkers. Traditional prognostic/predictive factors in breast cancer include tumor size, grade, lymph node status, hormone receptor (ER and PR) status, vascular invasion, and age [237]. A number of biological factors that relate to tumor aggressiveness or metastatic potential, including markers of angiogenesis and proliferation, growth factor receptors, cell cycle regulators, and proteases, have been discovered [237].

A number of kallikreins were shown to be putative prognostic or predictive breast cancer markers [42]. The expression of *KLK5* and *KLK14* in breast tumors are indicative of a poor patient prognosis [93, 94], whereas higher levels of *KLK9*, *KLK13*, and *KLK15* mRNA and the hK3 protein forecast a favorable disease outcome [89, 155, 215, 238]. Furthermore, high levels of hK3 and hK10 proteins in breast carcinomas are significantly related to a poor response to tamoxifen therapy [239]. More recently, our *in silico* analysis showed downregulation of at least four kallikrein genes (*KLK5*, *KLK6*, *KLK8*, and *KLK10*) in breast cancer [240]. The potential diagnostic and prognostic roles of kallikreins in breast cancer are summarized in Table 6.

### 16.2.3. Prostate Cancer

Prostate cancer is the most commonly diagnosed tumor in American men, accounting for 33% (220,900 cases) of all male cancers [222]. Several kallikreins have diagnostic, prognostic, or predictive values in prostate carcinoma (Table 7). Lower tissue hK3 concentration is associated with more aggressive forms of this cancer [241]. High *KLK5* and *KLK11* mRNA levels also indicate a favorable prognosis [169, 242], whereas *KLK14* and *KLK15*

overexpression indicates an unfavorable prognosis for prostate cancer patients [51].

#### 16.2.4. Testicular Cancer

We have recently published a review documenting the apparent relationship between kallikreins and testicular cancer [92]. The *KLK5* gene has potential as a favorable prognostic marker for testicular cancer patients [243]. Furthermore, the differential expression of *KLK10* and *KLK14* and *KLK13* splice variants in testicular cancer tissues have been recently reported [172, 199, 244]. The potential diagnostic or prognostic roles of kallikreins in testicular cancer are summarized in Table 8. Further studies are clearly warranted.

#### 16.2.5. Lung, Pancreatic, and Colon Cancers and Leukemias

A microarray study has identified at least one kallikrein gene being overexpressed in lung carcinoma (*KLK11*), particularly in neuroendocrine tumors [245]. Because many kallikreins are coexpressed in normal lung tissue [43], we hypothesize that multiple kallikreins, in addition to *KLK11*, may also be deregulated in lung cancer.

Expression of multiple kallikrein genes was reported in endocrine and exocrine pancreas by immunohistochemistry [81–83]. More recently, our *in silico* analysis, using two independent databases of the Cancer Genome Anatomy Project, provided evidence that some kallikreins are differentially regulated in pancreatic cancer [246]. In particular, *KLK6* and *KLK10* were significantly upregulated. This finding is in accord with recent data from microarray analysis [247].

We have also recently provided evidence indicating the overexpression of three kallikreins (*KLK7*, *KLK8*, and *KLK10*) and downregulation of another kallikrein (*KLK1*) in colon cancer [246]. A recent report showed also a downregulation of the *KLK10* gene in acute lymphoblastic leukemia [143].

#### 16.2.6. Brain Tumors

The possible involvement of kallikreins in brain tumors has been examined recently. A recent report showed expression of the hK6 protein by glioblastoma cells implanted intracranially in nude mice. Moreover, hK6 expression was shown to colocalize with the expression of an invasion-associated matrix-cellular protein called SPARC [248]. Given the high level of expression of some kallikreins in the brain, it is logical to speculate a possible involvement of kallikrein in brain tumors. Ongoing studies are now being conducted to evaluate the possible functional importance of kallikreins in brain tumor invasiveness [248].

### 16.3. HOW ARE KALLIKREINS INVOLVED IN CANCER?

Several mechanisms can be proposed by which kallikreins can be involved in the pathogenesis of endocrine-related malignancies. Proteolytic enzymes are thought to be involved in tumor progression because of their role in extracellular matrix degradation. Many studies have shown that a variety of proteolytic enzymes are overproduced either by the cancer cells themselves or by the surrounding stromal cells, and that their overexpression is associated with unfavorable clinical prognosis [249].

Breast, prostate, testicular, and ovarian cancers are all considered “hormonal” malignancies. Sex hormones are known to affect the initiation or progression of these malignancies. However, all kallikreins are under sex steroid hormonal regulation. Taken together, kallikreins may represent downstream targets by which hormones affect the initiation or progression of such tumors.

Experimental evidence indicates that hK2 and hK4 can activate the proform of another serine protease, the urokinase-type plasminogen activator [106, 162]. Urokinase activates plasmin from its inactive form (plasminogen), which is ubiquitously located in the extracellular space, leading to degradation of the extracellular matrix proteins. This might provide some clues about the role of kallikreins in cancer progression and could explain the differential expression of several kallikreins in tumors. Plasminogen can also activate precursor forms of collagenases, thus promoting the degeneration of the collagen in the basement membrane surrounding the capillaries and lymph nodes. Another kallikrein, hK7, can degrade the alpha chain of human fibrinogen, and it is hypothesized to be involved in an apoptotic-like mechanism that leads to desquamation of the skin [166]. The involvement in growth and apoptotic activities is also reported for hK3, which can digest insulin-like growth factor-binding protein 3 [250] and parathyroid hormone-related protein [251]. Similar findings were observed for some rodent kallikreins [252].

Bhoola *et al.* [163] have recently provided strong evidence indicating the presence of hK1 activity in the chemotactically attracted inflammatory cells of esophageal and renal cancers, indicating a role for kallikreins in these malignancies. Modulation of angiogenic activity is another possible mechanism for kallikrein involvement in cancer. The kinin family of vasoactive peptides, liberated by hK1 action, is believed to regulate the angiogenic process [253]. It was recently reported that immunolabeling of hK1 was intense in the angiogenic endothelial cells derived from mature corpora lutea. Immunoreactivity was lower in nonangiogenic endothelial cells, and least in angiogenic endothelial cultures of the regressing corpus luteum [253]. In addition, hK3 was reported to have antiangiogenic activities [254].

The elevation of serum concentration of kallikreins in cancer might be a result of the increased vasculature (angiogenesis), the destruction of the glandular architecture of the tissues involved, and the subsequent leakage of these proteins into the general circulation. It is possible that the concentration of kallikreins may also be increased in serum because of gene overexpression.

## **17. Therapeutic Applications**

It is possible that some kallikreins may become valuable therapeutic targets when the biological pathways that are involved are delineated. For example, the enzymatic activity of these serine proteases may initiate (e.g., tumor invasion, activation of hormones, growth factors, other enzymes, receptors or cytokines, amyloid formation) or terminate (e.g., inhibition of angiogenesis, inactivation of growth factors, hormones, enzymes, cytokines, or receptors) biological events. Once known, these events could be manipulated, for therapeutic purposes, by specific enzyme inhibitors or activators. Another potential therapeutic approach is the cell-specific activation of therapeutic agents [255]. Preliminary reports show potential success by using the PSA promoter to express molecules in a tissue-specific fashion [256]. A third possible therapeutic approach involves immunotherapy or development of cancer vaccines. With our increasing knowledge of the hormonal regulation of kallikreins, hormonal activation (or repression) of kallikrein activity could be investigated in the future.

## **18. Future Directions**

The kallikrein locus in humans has now been well characterized and confirmed by independent analyses of various investigators. The structure of these simple serine protease genes has now been fully elucidated. It is thus now appropriate to shift the research focus from characterization of the gene structure to study of the functional aspects of kallikreins in humans and other species. The completion of the mouse, rat, and other genome sequences will aid in comparative genomic analysis of the human and other animal kallikrein loci, toward establishing phylogenetic and functional relationships. The large number of kallikrein genes and proteins and their close localization point to an important proteolytic system that has not yet been recognized. We do not, therefore, know whether the tight clustering of these genes is related to their physiological functions or whether it represents a functional redundancy.

With very few exceptions, knockout animal models for kallikreins have not yet been developed. It will be very interesting to establish the phenotype of mice lacking not one but many kallikreins, or even the whole-mouse kallikrein locus. This will provide insights into the function of these genes in the mouse and further delineate the degree of redundancy of these genes. Mutational analysis of human kallikreins has not been performed in detail, and we do not yet know whether the functional inactivation of any of these genes in humans leads to recognizable diseases.

The localization of these genes next to each other and their parallel expression in many tissues strongly indicate that these genes are regulated by a locus-control region. This proposal merits further investigation. However, epigenetic changes seem to play a role in kallikrein gene silencing. Furthermore, kallikrein genes are regulated by steroid hormones and vitamin D in certain tissues. A better understanding of their mode of regulation will be important in the future.

It appears that small groups of kallikreins may represent enzymatic cascade pathways in certain tissues. For example, it is very likely that at least three kallikreins, hK2, hK3, and hK11, which are present in seminal plasma at relatively very large concentrations, may coordinately act as a cascade enzymatic pathway, involved in semen liquefaction or other activities. In contrast, another group of kallikreins, including hK5 and hK7 and possibly many others, seem to be involved in skin desquamation. Similar cascade pathways may be operating in the breast, testis and other tissues.

Some avenues for future kallikrein research include, first, their continued investigation as promising novel biomarkers for diagnosis, prognosis, and monitoring of many diseases, particularly cancer. Although most of the current reports describe single kallikreins as potential biomarkers, in the future, it may be possible to combine multiple kallikreins with other tumor markers in multiparametric panels. However, the recognition that kallikrein genes give rise to a very large number of splice variants (more than 70) offers new avenues of investigation regarding the applicability of these molecules as cancer biomarkers. It is possible that splice variants of some of these genes may be even more promising cancer biomarkers than the classical forms of the enzymes.

Second, the full utilization of the kallikrein gene family in various aspects of human physiology and pathobiology will necessitate the delineation of their physiological functions. Future investigations should include the examination of their enzymatic specificity and their regulation by specific or nonspecific tissue or circulating inhibitors. We are currently using synthetic peptide substrates, combinatorial substrate libraries, macromolecular protein substrates, and phage display technology to delineate the physiological function of these enzymes.

Third, it is likely that these enzymes participate in cascade enzymatic reactions, similar to those of the coagulation cascade, in which one enzyme activates or inactivates another. Further understanding of these interrelationships between kallikreins and possibly other proteolytic systems (such as the metalloproteases) will further aid in the elucidation of their functions. Because these enzymes are all predicted to be secreted, it is likely that they act in the intercellular space by either cleaving cell surface receptors or participating in the remodeling of extracellular matrix. These activities may lead to the discovery of novel signal transduction pathways associated with cell adhesion, remodeling, or angiogenesis.

Fourth, the demonstration that many of these enzymes have prognostic value in cancer may qualify them as novel therapeutic targets. For example, similar to many other proteases, these enzymes may participate in the digestion of extracellular matrix, thus facilitating tumor invasion and metastasis. The identification of highly specific inhibitors may reveal new therapeutic opportunities.

Fifth, certain kallikreins, such as human kallikrein 6, are highly expressed in the central nervous system. It has previously been shown that hK6, and possibly some other kallikreins, are implicated in inflammatory reactions within the central nervous system that lead to demyelination. The association of hK6 and some other kallikreins with AD and multiple sclerosis points to the possibility that some of these enzymes may play important roles within the central nervous system. In addition, many of these enzymes have been found in endocrine tissues such as the islets of Langerhans, thyroid, pituitary, and others, pointing to the possibility that they may participate in prohormone or hormone processing.

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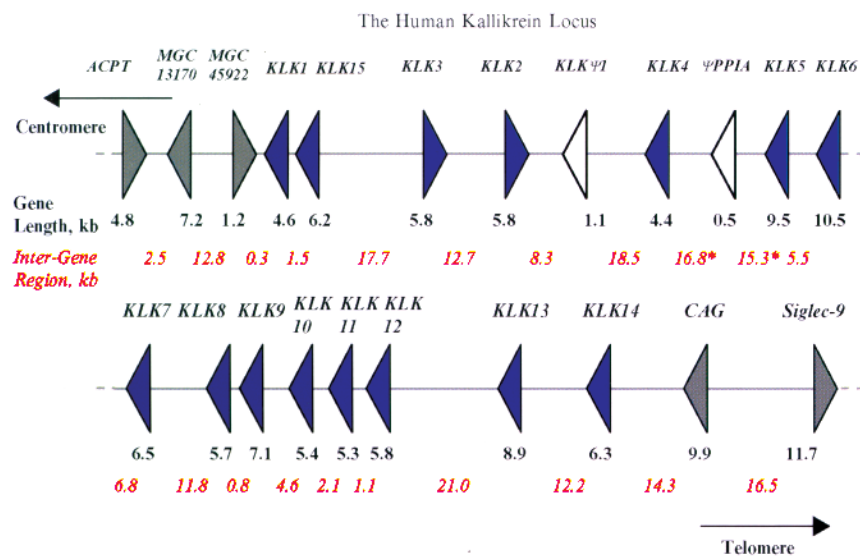
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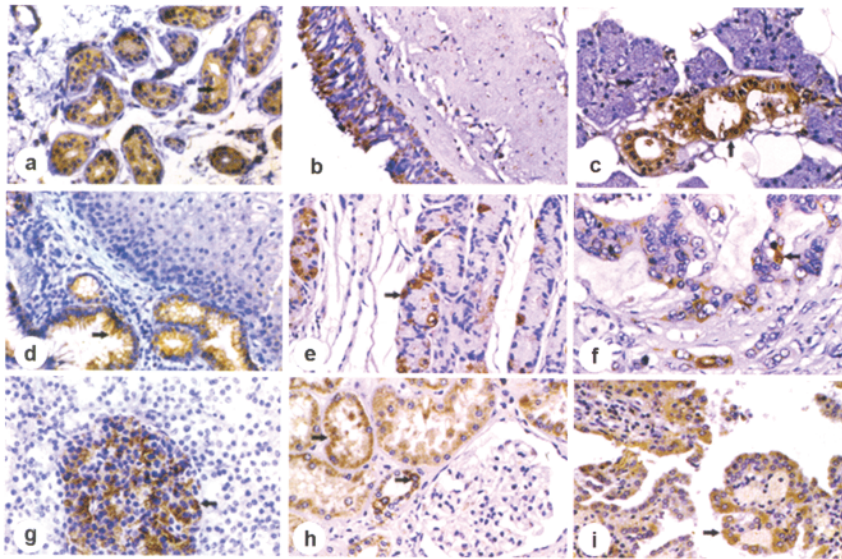


YOUSEF *ET AL.*, FIG. 1. Schematic representation of the human kallikrein gene locus on chromosome 19q13.4. Genes are represented by arrowheads indicating the direction of transcription. Kallikrein genes are shown in blue, and nonkallikrein genes and pseudogenes are presented by grey and white arrows, respectively. Official gene symbol is shown above each gene, and the approximate gene length is shown below each gene in kilobytes. The approximate intergenic regions are shown in red in Kb. CAG, cancer associated gene (GenBank accession AY279382); ACPT, testicular acid phosphatase (GenBank accession AF321918). The position of the PPIA pseudogene is provisional and the length of the intergenic regions (shown with asterisks) may change in the future. MGC, mammalian gene collection; these two genes have not as yet been characterized.

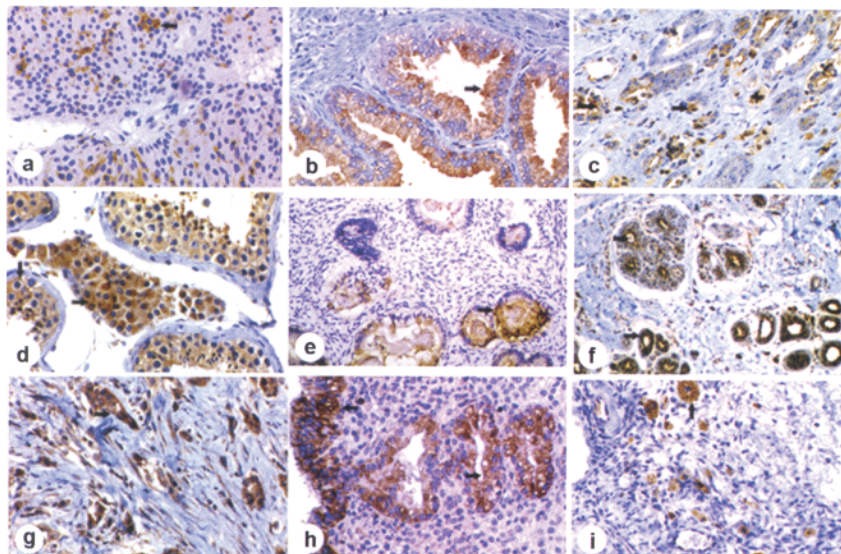
	KLK1	KLK2	KLK3	KLK4	KLK5	KLK6	KLK7	KLK8	KLK9	KLK10	KLK11	KLK12	KLK13	KLK14	KLK15
ADIPOSE	Red	Yellow	Yellow	Green	White	Green	Green	Yellow	White	Yellow	Yellow	Yellow	Yellow	Yellow	Orange
ADRENAL GLAND	Orange	Yellow	Yellow	Orange	White	Yellow	White	Yellow	White	Green	White	White	Yellow	Yellow	Green
BONE MARROW	Green	Yellow	Yellow	Yellow	Green	Yellow	Orange	Orange	Orange	Green	Green	White	Yellow	Yellow	White
CEREBELLUM	Yellow	Yellow	Yellow	Green	Green	Orange	Red	Green	White	Red	Green	Yellow	Yellow	Orange	Yellow
ADULT BRAIN	Yellow	Yellow	Yellow	Yellow	Green	Orange	Green	Yellow	Orange	Orange	Green	Yellow	Yellow	Orange	Yellow
CERVIX	Green	Yellow	Yellow	Orange	Orange	Orange	Orange	Red	Orange	Red	Red	Yellow	Orange	Green	White
COLON	Red	White	Yellow	Green	Yellow	Yellow	White	Yellow	White	Orange	Green	Yellow	Yellow	Yellow	Red
ESOPHAGUS	Yellow	Yellow	Yellow	White	White	Yellow	Orange	Red	Green	Red	Green	Orange	Green	White	White
FALLOPIAN TUBE	Yellow	Yellow	Yellow	White	White	Green	Orange	Orange	Orange	Orange	Yellow	Orange	Yellow	Yellow	White
FETAL BRAIN	Green	White	Yellow	Yellow	Yellow	Orange	White	White	White	Orange	Green	White	Green	Green	White
FETAL LIVER	Green	White	Yellow	White	Yellow	Green	Yellow	Yellow	White	Yellow	Green	Yellow	White	Green	White
HEART	Green	White	Yellow	White	Yellow	Green	Yellow	Yellow	White	Yellow	Yellow	White	Yellow	Yellow	White
HIPPOCAMPUS	White	White	White	White	White	Orange	Green	Yellow	White	Yellow	White	White	Yellow	Yellow	White
KIDNEY	Red	Yellow	Yellow	White	White	Orange	Red	Green	Yellow	Yellow	Yellow	Yellow	Yellow	Green	Yellow
LIVER	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White
LUNG	Green	White	Yellow	White	White	White	Orange	Green	White	Red	Red	Yellow	Green	Yellow	White
MAMMARY GLAND	Orange	Green	Green	Yellow	Green	Orange	Red	Red	Yellow	Red	Red	Yellow	Green	Red	White
OVARY	Green	White	White	White	White	Orange	Orange	Orange	White	Red	Green	Yellow	Yellow	Green	White
PANCREAS	Red	White	White	White	White	White	White	White	White	Yellow	Red	Yellow	Green	Green	Yellow
PLACENTA	Yellow	White	Yellow	White	White	Yellow	Green	Green	White	Yellow	Yellow	White	White	Yellow	White
PROSTATE	Red	Red	Red	Green	Yellow	Yellow	Green	Green	Yellow	Red	Orange	Green	Yellow	Orange	Orange
PITUITARY	White	White	White	White	White	White	White	White	White	Yellow	Green	Yellow	White	Yellow	White
SALIVARY GLAND	Orange	Green	Green	Red	Orange	Orange	Orange	Orange	White	Orange	Red	Red	Green	Yellow	Orange
SKELETAL MUSCLE	Yellow	Yellow	Yellow	Yellow	White	White	White	Yellow	White	Yellow	Yellow	White	White	White	White
SKIN	Orange	Yellow	White	White	Yellow	Green	Red	Red	White	Green	Orange	White	Yellow	Yellow	White
SPINAL CORD	Green	Yellow	Green	Green	Yellow	Red	Orange	Orange	White	Green	Yellow	White	White	Green	Yellow
SPLEEN	Orange	White	Yellow	Yellow	Yellow	Orange	Orange	Orange	White	Yellow	Yellow	White	White	Green	White
SMALL INTESTINE	Orange	White	Yellow	Yellow	Yellow	White	Green	Yellow	White	Green	Green	Yellow	Yellow	Green	White
STOMACH	Green	White	Yellow	Yellow	Green	Orange	Orange	Orange	Red	Orange	Orange	Orange	Yellow	Green	White
TESTIS	Green	Green	Green	Orange	Red	Orange	Orange	Red	Yellow	Orange	Red	Yellow	Green	Red	Green
THYMUS	Green	Yellow	Yellow	Yellow	Yellow	Green	Green	Yellow	Yellow	Green	Green	Yellow	Yellow	Orange	Yellow
THYROID	Green	Yellow	White	Orange	Yellow	Orange	Orange	Orange	White	Orange	Orange	Orange	Green	Green	Green
TONSIL	Green	White	White	White	White	Yellow	Green	Red	Yellow	Green	Green	White	Green	White	Yellow
TRACHEA	Green	Yellow	Yellow	Yellow	White	Green	Orange	Orange	Yellow	Orange	Red	Orange	Orange	Green	Yellow
UTERUS	Yellow	White	Yellow	White	White	White	White	White	White	Green	Yellow	Yellow	White	White	White
VAGINA	Red	White	Yellow	Yellow	Red	Green	Red	Red	Red	Red	Red	Green	Red	Red	Yellow

SEMIQUANTITATIVE EXPRESSION SCORING SYSTEM					
Very High	High	Moderate	Low	Very Low	Nothing

YOUSEF *ET AL.*, FIG. 2. Expression map of human tissue kallikreins in a variety of tissues, as determined by reverse transcriptase polymerase chain reaction. The relative semiquantitative expression levels for each gene are indicated.

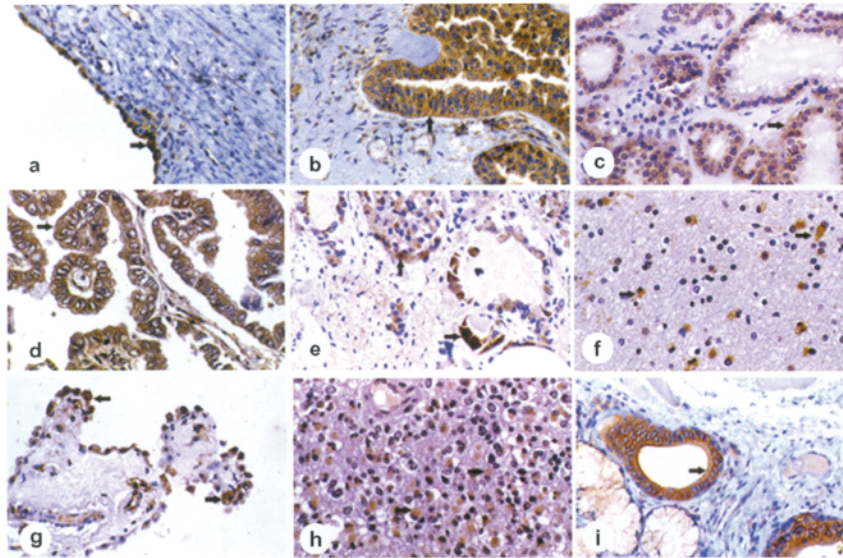


YOUSEF *ET AL.*, FIG. 3. Immunohistochemical expression of (a) hK7 by the epithelium of eccrine glands of the skin (monoclonal antibody, clone 73.2), (b) hK13 by the epithelium of the bronchus (monoclonal antibody, clone IIC1), (c) hK5 by the ductal epithelium of the parotid gland (polyclonal antibody), (d) hK7 by the esophageal glands (monoclonal antibody, clone 73.2), (e) hK13 by the gastric mucosa (monoclonal antibody, clone 2-17), (f) hK6 by the large intestine mucosa (polyclonal antibody), (g) hK10 in an islet of Langerhans in the pancreas (monoclonal antibody, clone 5D3), (h) hK11 by the epithelium of the urinary tubuli (monoclonal antibody), (i) hK11 by a papillary renal cell carcinoma (monoclonal antibody).



YOUSEF *ET AL.*, FIG. 4. (*continues*)





YOUSEF *ET AL.*, FIG. 5. Immunohistochemical expression of (a) hK14 by the ovarian surface epithelium (polyclonal antibody), (b) hK14 by a cystadenocarcinoma of the ovary (polyclonal antibody), (c) hK10 by hyperplastic follicles of the thyroid gland (monoclonal antibody, clone 5D3), (d) hK6 by a papillary thyroid carcinoma (polyclonal antibody), (e) hK13 by endocrine cells in the pituitary gland (monoclonal antibody, clone 2-17), (f) hK10 by glial cells in the brain (monoclonal antibody, clone 5D3), (g) hK13 by the choroid plexus epithelium (monoclonal antibody, clone 2-17), (h) hK5 by a glioma (monoclonal antibody, clone 6.10), (i) hK7 by the ducts of the submucosal glands of the tonsils (monoclonal antibody, clone 85.2).

YOUSEF *ET AL.*, FIG. 4. (*Continued*) Immunohistochemical expression of: (a) hK10 by a low-grade urothelial carcinoma (monoclonal antibody, clone 5D3), (b) hK11 by the secretory epithelium of the prostate gland (polyclonal antibody), (c) hK11 by a Gleason score 6 prostate carcinoma (polyclonal antibody), (d) hK14 by the spermatic epithelium and the stromal Leydig cells in the testis (polyclonal antibody), (e) hK10, by epithelial elements in a testicular immature teratoma (monoclonal antibody, clone 5D3), (f) hK14 by lobuloalveolar structures of the breast (polyclonal antibody), (g) hK14 by a ductal breast carcinoma, grade II (polyclonal antibody), (h) hK13 by the glandular epithelium of the endometrium (polyclonal antibody), (i) hK14 by luteinized stromal cells of the ovary (polyclonal antibody).