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Human tissue kallikreins as prognostic biomarkers and as potential targets for anticancer therapy

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The tissue kallikrein family represents the largest cluster of contiguous proteinase genes in the entire human genome. Its 15 members are expressed primarily in a tissue-specific manner, but can also be detected in biological fluids such as serum and seminal plasma. The mature active tissue kallikreins play vital roles in numerous physiological and pathological processes, in which they are able to function individually or in cascade pathway(s). Their genetic polymorphisms, alternative splicings and aberrant amounts of transcripts and/or proteins are often correlated with increased cancer risk, thus providing a sensible application for the use of tissue kallikreins as diagnostic, prognostic and predictive tumour markers. In this review, an updated overview of scientific research and patents regarding the functional features and clinical indications of tissue kallikreins in neoplastic diseases is provided.

Keywords: kallikrein, metastasis, prognosis, proteinase, proteolytic enzyme, serine proteinase, tumour marker

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1. Introduction

Ever since the first proteolytic enzyme was reported in the 19th century [1], thousands of this type of proteins have been identified that function to catalyse the hydrolysis of peptide bonds [2-4]. Approximately 1 – 5% of the genes are now estimated to encode for proteolytic enzymes, irrespective of the organism source [5-6,201]. In humans, > 550 known and putative proteolytic enzymes were recognised to act as regulators in many physiological processes [5-8,201-202]. These enzymes are classified based on three major criteria: i) the position of the scissile peptide bond within the substrate; ii) the catalytic mechanism of action; and iii) the evolutionary relationships of protein structures [5,6,201]. Under the first criterion, each proteolytic enzyme is categorised into one of the two following types: i) exopeptidases (or peptidases) that hydrolyse peptide bonds from the ends of a protein or polypeptide chain to produce tiny peptides and amino acids; and ii) endopeptidases (or proteinases) that cleave inside the target substrate to produce large peptides [5-8,201]. According to the second criterion, members of the exopeptidases are divided into several classes, for example, aminopeptidases cleave amino acids from the N-terminal end, whereas carboxypeptidases remove amino acids from the C-terminal site. For endopeptidases, a total of six mechanistic classes, including serine, metallo-, cysteine, aspartic, threonine and glutamic proteinase are distinguished based on the main functional residue or metal ion involved in the catalytic reaction [6,9,201]. With regard to the third criterion, proteolytic enzymes can be further specified into different clans with each clan

consisting of various families whose members have evolved from a common ancestor [6,201].

Serine proteinases are the most widespread and extensively studied class of proteolytic enzymes [10,11]. Numerous publications and patents have described the use of serine proteinases in a wide range of industrial applications, including textile formulations, detergent additives, food processing, cosmetic treatments and medical therapies. Serine proteinases contain a nucleophilic Ser residue at the active site that is critical for proteolytic activity [10,11]. While the majority of serine proteinases are equipped with a classical Ser-His-Asp catalytic triad (or called charge-relay system) to help through relocation of proton charge to break the peptide bond, few have been identified to possess novel catalytic triads, such as Ser-His-Glu with glutamic acid replacing the usual aspartate [12-16]. Two evolutionary groups are represented in this class of endopeptidases: the mammalian-type (i.e., trypsins, chymotrypsins, kallikreins and elastases) and the bacterial-type serine proteinases (i.e., subtilisins and sedolisins) [6,201]. These two groups, even though they are not related in sequence or structure, share a very similar mechanism of action and provide one of the most remarkable examples at the molecular level of convergent evolution. Serine proteinases are made up by at least 15 different clans with the medically most interesting one being the PA clan [6,201]. The PA clan, especially its S1 family, is involved in a variety of normal and pathological processes, including digestion, blood coagulation, fibrinolysis, complement activation, allergy and inflammatory responses. In the case of cancer diseases, members of the S1 family were found to play significant roles in tumour growth, invasion and metastasis [17-21]. These proteolytic enzymes have emerged as attractive candidates for use as therapeutic targets and as diagnostic and/or prognostic markers in cancer. This article will review the biological aspects in particular of kallikreins, a subgroup of the S1 family of serine proteinases, and their potential as tumour biomarkers and as targets for anticancer therapy.

2. Kallikrein serine proteinases

The term 'kallikrein' was first introduced by Werle and colleagues who detected high levels of a substance's activity in the pancreas (in Greek word, the 'kallikreas') [22,23]. The substance is now known as tissue kallikrein-1 (KLK1), an endopeptidase that releases Lys-bradykinin by limited proteolysis of kininogen 1. Two distinct types of kallikreins have been identified to date: the circulating liver-synthesised plasma kallikreins; and the KLK1 and its related serine endopeptidase [6,201]. These kallikreins include both regulatory- and degradative-type proteolytic enzymes that participate in various physiological activities such as cellular proliferation, extracellular matrix (ECM) degradation, tumourigenesis, prohormone processing, tissue remodelling and blood pressure regulation [24]. Interestingly, while plasma kallikrein (*KLKB1*) occurs as a sole gene on human chromosome 4q35, *KLK1* and its 14 related genes (*KLK2* – *KLK15*) are localised in tandem on chromosome 19q13.4 and form the largest cluster of contiguous proteinase genes in the entire genome (**Figure 1A**) [20,21,25-28,101-113]. The ~ 300 kb *KLK* locus, with no intervention by any non-*KLK* genes, is bound centromerically by the testicular acid phosphatase gene (*ACPT*) and telomerically by the sialic acid-binding immunoglobulin-like lectin 9 gene (*SIGLEC9*). The 3 classical members (*KLK1, KLK2* and *KLK3*) are grouped together within an ~ 60 kb region with *KLK15*, whereas *KLK4* – *KLK14* and the Ψ*KLK1* pseudogene are clustered telomeric to *KLK2* (**Figure 1A**). These *KLK* genes are in the range of ~ 4 – 10 kb in length and possess highly conserved gene organisation pattern (i.e., exon-intron phases), with 5 coding exons that are very similar both in size and sequence (**Figure 1B**) [26-28]. The start codon for the *KLK* genes is generally located 8 – 87 bp from the end of coding exon 1 and the stop codon is situated at ~ 150 – 189 bp from the start of coding exon 5. The identification, mapping, molecular cloning and expression profiling of some of these *KLK* genes have been claimed in ~ 13 patents granted around the world, with few featuring in separate patents [101-113].

KLKs are synthesised as secreted prepropeptides containing: i) a signal peptide (16 – 33 amino acid residues) that directs them to the endoplasmic reticulum for secretion; ii) a pro-peptide (4 – 9 amino acid residues except KLK5) that maintains them as inactive precursors (zymogens); and iii) a chymotrypsin-like (KLK3, KLK7, KLK9) or trypsin-like (KLK1, KLK2, KLK4 – KLK6, KLK8, KLK10 – KLK15) domain responsible for catalytic activity (**Figure 1C**) [20,21,28]. The pro- and mature KLK proteins are formed from the sequential proteolytic cleavage of the signal and pro-peptides on entry into the secretory pathway and on activation, respectively [28]. For mature KLK enzymes, they exhibit a typical structure of serine endopeptidases dominated by two juxtaposed six-stranded antiparallel β-barrels with the His-Asp-Ser catalytic triad bridging the barrels [12,29]. Among the 15 human KLK proteins, a maximum homology is found around the amino acids of the catalytic triad, including the histidine residue in the conserved fragment of WVLTAAHC, the aspartic acid residue in the segment of DLML and the serine residue in the region of GDSGGPLVC [28]. The number and position of all cysteine residues are also highly conserved, with the overall amino acid identity between various members of the KLK family in the range of ~ 40 – 80%.

As exemplified in **Figure 2** for the catalytic mechanism of the archetypal serine proteinase chymotrypsin, active KLK enzymes utilise Ser195 (chymotrypsin numbering) to attack the carbonyl carbon atom of the scissile peptide bond of the substrate, making the carbonyl group of the amide bound converted into a negatively charged carbonyl oxygen (CO). Thus, the oxygen carries a negative net charge, which allows the formation of the first tetrahedral transition state. The resulting $His57^{-}H^{+}$ is stabilised by the hydrogen bond to Asp102. The oxyanion of the tetrahedral intermediate is

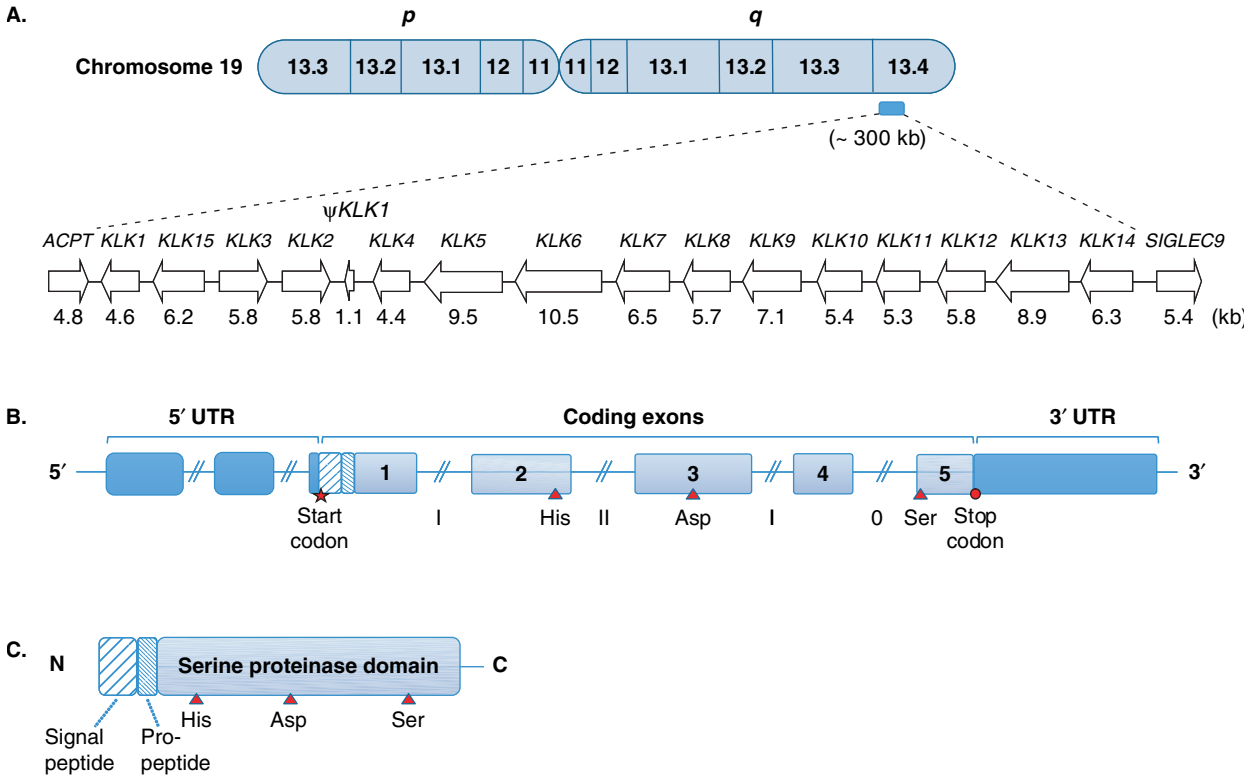

Figure 1. Gene locus, organisation pattern and protein characteristics of the human tissue kallikrein family. A. The *KLK* locus spans ~ 300 kb on the q arm of chromosome 19 in cytogenic region 13.4. This locus is bound centromerically by the testicular acid phosphatase gene (*ACPT*) and telomerically by the sialic acid-binding immunoglobulin-like lectin 9 gene (*SIGLEC9*). The 15 *KLK* members are clustered in a tandem array with no intervention by any non-*KLK* genes. Arrowheads show the location of genes and their direction of transcription. Except *KLK2* and *KLK3*, the direction of *KLK* gene transcription is from telomere to centromere. Official gene names are abbreviated to their numbers and indicated above each arrowhead, whereas the lengths of the genes are shown in approximate kilobases beneath each arrowhead. Note that the lengths between different *KLK* members are not drawn to scale. **B.** The 15 *KLK* genes, are in the range of ~ 4 – 10 kb in length, possess highly conserved organization pattern with 5 coding exons that are similar both in sequence and size and 4 intervening introns that share a conserved intron phase pattern (I, II, I, 0). The positions of the codons for the catalytic triad (shown in triangles) are also conserved, with the codon for His residue near the end of exon 2, the codon for Asp residue in the middle of exon 3 and the codon for Ser residue near the start of exon 5. Most *KLK* genes have one or two non-coding exons in the 5′ untranslated region (5′ UTR). The 3′ UTR typically varies in length. The asterisk represents the start codon and the dot is for the stop codon. **C.** The 15 KLK members are thought to be transcribed and synthesised as secreted prepropeptides containing a signal peptide (16 – 30 amino acid residues) that directs them to the endoplasmic reticulum for secretion, a pro-peptide (4 – 9 amino acid residues) that maintains them as inactive precursors (zymogens) and a serine-proteinase domain responsible for catalytic activity. The residues for the classical His-Asp-Ser catalytic triad are shown as triangles.
The figure is modified from refs. [21] and [63].

stabilised by interaction with the main chain NHs of the oxyanion hole. The tetrahedral intermediate collapses with expulsion of the leaving group, helped by $His57^{-}H^{+}$ acting as a general acid, to yield the acyl enzyme intermediate. The amine portion of the substrate is dissociated and replaced with a water molecule. The imidazole nitrogen contributes to polarisation of the water molecule, then in turn attacks the carbonyl carbon of acyl enzyme followed by the formation of the second tetrahedral intermediate. At the end of the reaction, the deacylation leads to reconstruction of the carboxyl group in the hydrolysed substrate, which is concerted with the release of an active KLK enzyme (**Figure 2**). Notably, while all other KLK serine proteinase are secreted enzymes with extracellular functions, KLK4 is primarily localised to the nucleus, indicating that this serine proteinase may have a different function compared with other members of the KLK family. By utilising a positional-scanning combinatorial library of tetrapeptide substrates, Debela and colleagues have recently endeavoured to determine the substrate specificity of seven KLK members [30]. Both the KLK3 and KLK7 endopeptidases were shown to possess a chymotrypsin-like specificity preferring large hydrophobic or polar residues at the P1-position. The KLK4, KLK5 and less stringent KLK6 proteolytic enzymes demonstrated a trypsin-like specificity with strong preference for P1-Arg, whereas KLK10 and KLK11 exhibited

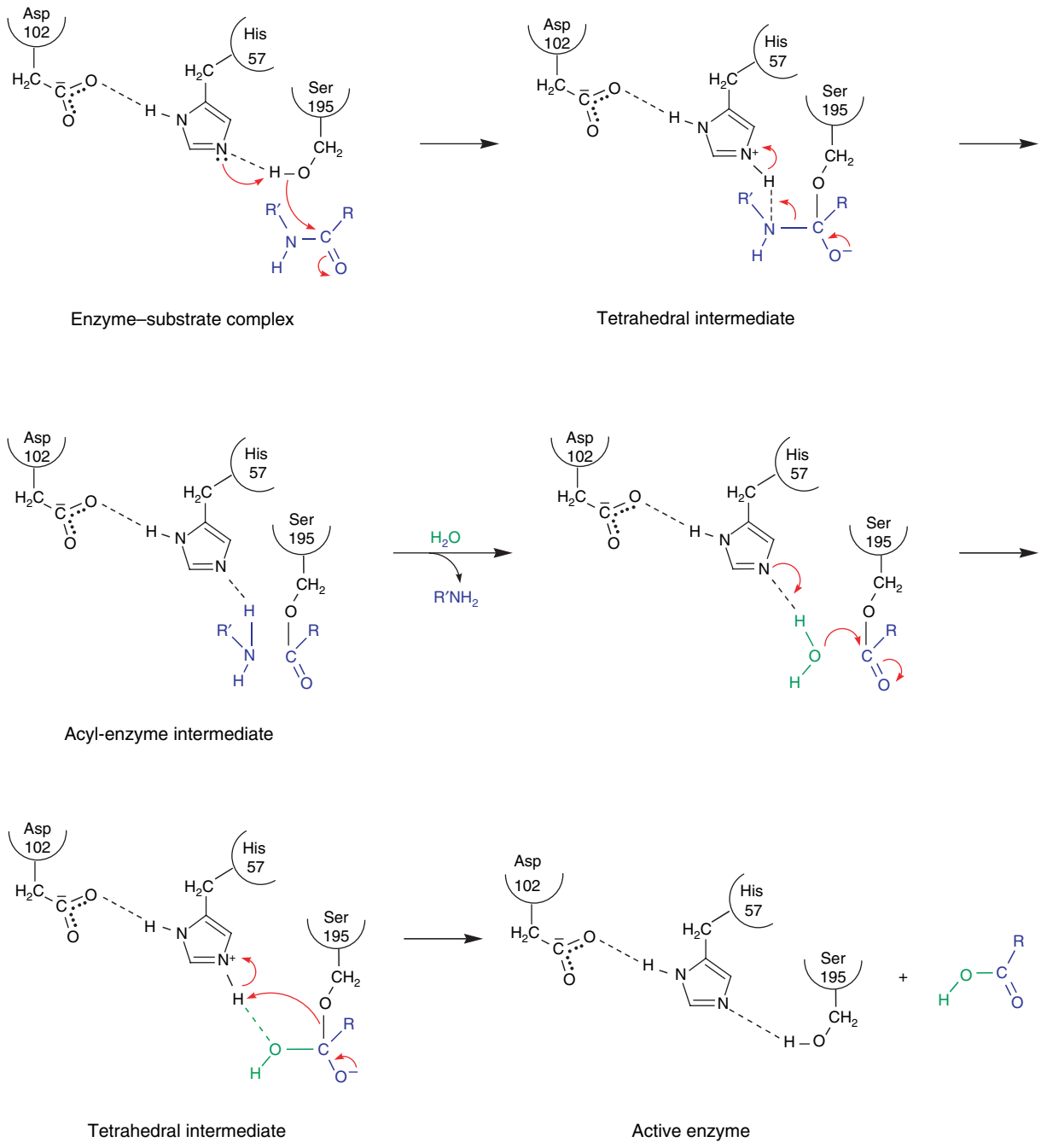

Figure 2. Catalytic mechanism of tissue kallikrein proteinase as exemplified by chymotrypsin (description in the text).

an ambivalent specificity, accepting both basic and large aliphatic P1 residues [30].

To date, dozens of splice variants have been reported for human *KLKs*, with most occurring in coding regions (i.e., skipping of exons 2 and 4) and few in non-coding regions (i.e., retention of intron III or splicing within the 5′ untranslated region) [31,32]. Despite the large amount of alternative mRNA transcripts identified, only a very small number have been confirmed at the protein level. For example, although > 50% of *KLK* splice variants were estimated to code for novel proteins, many of them would be truncated due to an open reading frame shift. In other cases, it was found that one or more residues of the Ser-His-Asp catalytic triad are missing, thus resulting in a severe hindrance or even complete loss of proteolytic activity [31,32]. Notably, in many *KLK* splice variants the sequence encoding the signal peptide is retained. It suggests that most *KLK* splice variants, on successful translation, are likely to be secreted and present

in biological fluids and may have clinical relevance in biomarker development and application [31,32]. For other splice variants occurring in the untranslated regions, it is believed that alterations within these regions may have an effect on post-transcriptional regulation such as mRNA stability, localisation and translational repression or activation.

3. Tissue specificity and transcriptional regulation of human tissue kallikreins

Most *KLK* genes show a relatively broad tissue distribution pattern, with the highest expression levels in certain tissues and lower levels in many others [20,21,33,34]. The KLK proteins can also be detected in biological fluids such as sweat, milk, serum, seminal plasma and cerebrospinal fluid [20,35]. With the newly developed ELISA assays for KLK proteins, Shaw and Diamandis recently examined global KLK abundance patterns in many human tissues and biological fluids. The results showed that KLK1 is most abundant in the pancreas, salivary gland and urine; KLK2, KLK3 and KLK11 are present mainly in the prostate and seminal plasma; KLK4 are in low concentrations in almost all tissues examined; KLK5, KLK7, KLK8 and KLK10 can be detected in high levels in skin and cervicovaginal fluid; KLK6 is most abundant in the brain, spinal cord and breast milk; KLK9 exists mainly in the heart and fetal cartilage; KLK12 is present in bone marrow and bone; KLK13 is at high levels in the esophagus, tonsil and cervicovaginal fluid; KLK14 is in fetal skin and cartilage; and KLK15 is most abundant in the breast, adult and fetal skin and fetal stomach [36]. When inside the cells, nearly all KLKs are localised primarily in the cytoplasm of glandular epithelia, from which they are secreted. Like their wild type forms, many *KLK* splice variants are also expressed in a tissue-specific manner, for example, alternative transcripts of *KLK2* and *KLK3* are highly expressed in the prostatic epithelium whereas those of the *KLK4, KLK8* and *KLK13* genes are present mostly in the skin [34]. Recent evidence suggests that different *KLK* genes, in particular *KLK2, KLK3, KLK4, KLK11* and *KLK15*, are often co-expressed within the same tissues, that is, prostate and salivary gland. However, the physiological and clinical relevance of the co-expression of these *KLK* genes remains unclear, and thus deserves additional investigation.

It is generally believed that the *KLK* expressions can be modulated by sex-steroid hormones especially in endocrine-related tissues. One typical example is the abundant expressions of *KLK2* and *KLK3* in the prostate in response to androgens and androgenic progestins [21,37,38]. Other *KLK* members such as *KLK5 – KLK7* and *KLK9 – KLK11* seem to be more responsive to estrogens [21,28,39,40]. In particular, *KLK4* was shown to be upregulated by androgens in prostate and breast tumour cells [41,42] and by estrogens in endometrial cancer cell lines [43]. A notable pattern related to the hormonal regulation is that the centromeric and telomeric groups of *KLK* genes (*KLK1 – KLK3*, *KLK13* and *KLK14*) on chromosome 19q13.4 are upregulated primarily by androgens, whereas the central group of the genes is upregulated mainly by estrogens [20,21]. Functional analyses on the *KLK* promoter regions and enhancers further revealed a complex transcriptional mechanism regulated by steroid hormone–receptor complexes. These complexes modulate the transcription of target *KLK* genes in either a direct or indirect fashion. In the former, the hormone-receptor complexes bind directly to the *cis*-acting sequences known as hormone response elements (i.e., estrogen or androgen response elements) within the proximal promoter and enhancer regions, hence recruiting necessary co-activators/repressors to stimulate *KLK* gene transcription [20]. In the indirect pathway, the hormone–receptor complexes modulate *KLK* expression via associations with *trans*-acting transcription factors. In addition, the control of *KLK* gene transcription may involve the integration of a myriad of transcription factors and signalling pathways that function to augment regulatory diversity to provide opportunities for cell and tissue-specific responses [44,45]. Up until now, research efforts have focused mostly on the transcriptional regulations of *KLK1 – KLK3* and *KLK10*. More functionally characterisations on the promoter/enhancer regions of other tissue-specific *KLK* genes are thus required to better understand the mechanisms and significances of the transcriptional regulations of the *KLK* genes.

4. Physiological roles of human tissue kallikreins

Despite their presence in various cell types and body fluids and by virtue of their proteolytic activity, the physiological roles of most KLK proteinases remain relatively obscure, except three classical members, KLK1, KLK2 and KLK3. KLK1 is the best functionally characterised member of the KLK family and is the only 'true tissue kallikrein' to cleave low molecular-weight kininogen to produce Lys-bradykinin, which in turn binds to its receptors, bradykinin B1 and B2, to mediate diverse physiological processes, including blood pressure regulation, neutrophil chemotaxis, vascular permeability, smooth muscle contraction and inflammatory cascades [20,21]. The X-ray crystal structure analysis revealed that KLK1 possesses unique dual-substrate specificity in hydrolysing low molecular-weight kininogen between both Arg-Ser and Met-Lys sequences. The binding of the peptide substrate further contributes to a structural rearrangement of the active-site Ser195 resulting in a catalytically competent juxtaposition with the active-site His57 [46]. Unlike KLK1, both KLK2 and KLK3 possess relatively low kininogenase activity [47,48]. Instead, KLK2 can cleave and activate itself [47-51] or hydrolyse pro-KLK3, although the latter remains largely in dispute. The mature active KLK2 and KLK3 enzymes then execute the proteolysis of seminal vesicle proteins, such as seminogelin I and II, and fibronectin in seminal fluid, hence resulting in increased sperm motility [52,53].

Other biological substrates, including peptide hormones, growth factor binding proteins and ECM components, were also identified for KLK2 and KLK3 (**Table 1**). Nonetheless, the diverse expression patterns of KLK1, KLK2 and KLK3 have led to the suggestion that the functional role of these serine proteinases may be specific to different cell types. Concerning the remaining KLK members, although their physiological roles have not yet been fully elucidated, some are thought to be involved in the processing of peptide hormones (KLK6 and KLK10), in the desquamation (KLK5, KLK7 and KLK8), in the CNS (KLK6 and KLK8) and in the degradation of ECM components (KLK5, KLK13 and KLK14) [47]. Furthermore, as all 15 *KLK* genes are clustered at the same chromosomal locus, their gene expressions and protein productions may be coordinately modulated by a common regulator (i.e., steroid hormones) to participate in sequential steps of the development of steroid hormone-driven neoplasia or in distinct proteolytic cascades such as blood coagulation, fibrinolysis, inflammatory response and ECM turnover [47]. The detailed contribution of the entire KLK family in these enzymatic cascade pathways should be further investigated.

5. Human tissue kallikreins as tumour biomarkers and as targets for anticancer therapy

Research on genetic polymorphisms in attempts to evaluate their use as susceptibility markers in various diseases has been growing rapidly over the past few years. Of > 10 million single nucleotide polymorphisms (SNPs) so far identified, several SNPs within the *KLK* family were found to be strongly correlated with cancer risk [20,21,54-61]. For example, one SNP at nucleotide 792 (C→T causing an active Arg226 wild type enzyme being substituted with an inactive Trp226 protein) of the *KLK2* coding region has been shown to result in much lower serum KLK2 levels and a higher risk of prostate cancer [54,57]. More significantly, at least 3 SNPs in the promoter region of *KLK3* at positions -158, -205 and -252 are closely related to breast and/or prostate cancer susceptibility [58,59]. For the SNP at position -158 (G→A), individuals homozygous for the G allele exhibited substantially increased KLK3 concentration in tumour cells and an increased overall survival rate than those homozygous for the A allele. The polymorphisms at positions -205 (A or AA) and -252 (G or A) were also shown to affect the transcriptional activity and the protein expression levels of KLK3, thus influencing cancer susceptibility [20,21,55]. The prevalence of other noteworthy SNPs, such as the one in coding exon 3 (G→A causing an Arg53His substitution) of *KLK1* and another at nucleotide 148 (G→T causing an Ala50Ser substitution) of *KLK10*, are all correlated with cancer risk due to significant decrease or complete loss of proteolytic activity [56-60]. These SNPs, in particular those of *KLK2* and *KLK3*, have been claimed in several patents by ExonHit Therapeutics for the use of detection, characterisation and/or treatment of cancers [114-117].

In addition to the genetic polymorphisms at the DNA level, the expressions of mRNAs and proteins of the *KLK* genes were frequently found to be differentially regulated in cancerous cells [20,21,62,63]. The detection of the presence of the aberrant KLK expression products, as well as their correlation with the risk of cancer have been claimed in several patents worldwide [118-123]. For instance, when compared with normal prostate tissues certain *KLK* genes (i.e., *KLK2, KLK3, KLK5, KLK6, KLK10* and *KLK13*) are downregulated, whereas others (i.e., *KLK4, KLK11* and *KLK15*) are upregulated in prostate cancer [21,64-72]. Such a correlation with tumour malignancy is not unexpected, as several KLK proteinases are able to degrade a variety of ECM protein components. Furthermore, one prerequisite for ECM degradation is that proteinases must encounter the matrix molecules in a microenvironment where they could maintain their proteolytic activity. This might occur intracellularly on phagocytosed matrix molecules or extracellularly after secretion of the KLKs.

Among all plausible candidates in prostate cancer susceptibility, *KLK3*, also named prostate specific antigen (PSA), has been used for > 20 years as an aid in the diagnosis and monitoring of prostate cancer. Lower serum concentration of the KLK3/PSA biomarker is associated with more aggressive forms of prostate cancer, such that tumour cells expressing high levels of KLK3/PSA are often related to a favourable prognosis [73,74]. Other *KLKs*, such as *KLK2, KLK5, KLK11, KLK14* and *KLK15*, have also been implicated as an adjuvant diagnostic marker for prostate cancer. The differential expressions of these KLKs may result in altered proliferation of prostate tumour cells, most likely through significant modifications in cell cycle regulatory gene expression. Thus, these *KLK* genes can aid in the differential diagnosis between benign prostatic hyperplasia and prostate cancer [70,75-77]. It is striking that 12 out of 15 *KLK* genes (*KLK2 – KLK8, KLK10, KLK11* and *KLK13 – KLK15*) are overexpressed in numerous ovarian carcinoma tissues, cell lines and/or serum and tumour ascites fluid. Preliminary clinical studies further support that overexpression of 11 KLKs in ovarian cancer is strongly associated with patient prognosis [20,21,64]. For instance, by using specific ELISAs with anti-KLK monoclonal antibodies, Diamandis and colleagues analysed hundreds of ovarian tumour extracts and attempted to correlate the amounts of different KLK proteins with clinicopathologic characteristics and patients outcome. Their results revealed that while several KLKs (i.e., KLK8, KLK11 and KLK13) have the potential to serve as either independent or dependent favourable prognosis indicators, two KLKs (i.e., KLK5 and KLK7) may in fact be markers of unfavourable prognosis for patients with ovarian cancer [78-82]. This paradox might likely be attributed to the dual role that the KLK family has during neoplastic progression, in that some members were found to promote while others can inhibit, directly or indirectly, tumour cell

Table 1. The human tissue kallikrein (KLK) family.

Name	Possible physiological substrates	Biomarkers for cancer detection and prognostication	Patent references
KLK1	LMW-kininogen, VIP angiotensinogen, Pro-MMPs	Not yet determined	[124,125,129]
KLK2	Pro-KLK3, seminogen I/II, IGFBPs, pro-uPA, fibronectin	Diagnostic and prognostic marker for breast and prostate cancers Marker for monitoring treatment response	[107,114-117,126-128,130,131]
KLK3	Seminogen I/II, IGFBPs, gelatin, laminin, fibronectin, ECM	Diagnostic and prognostic marker for breast, prostate and other cancers Marker for monitoring treatment response (i.e., tamoxifen)	[114-117,132,133]
KLK4	Pro-uPA, PAP	Unfavorable prognostic marker for ovarian cancer	[134]
KLK5	Corneodesmosin, fibrinogen	Unfavorable prognostic marker for breast and ovarian cancers Favorable prognostic marker for prostate and testicular cancers	[135,136]
KLK6	Collagens, laminin, fibronectin	Diagnostic and unfavorable prognostic marker for ovarian cancer	[137-139]
KLK7	Corneodesmosin, fibrinogen	Unfavorable prognostic marker for breast and ovarian cancers	[111,140,141]
KLK8	Myelin basic protein	Diagnostic and favorable prognostic marker for ovarian cancer Marker for monitoring treatment response	[142,143]
KLK9		Favorable prognostic marker for breast and ovarian cancers	[144,145]
KLK10		Predictive value for breast, cervical and prostate cancers Diagnostic and unfavorable prognostic marker for ovarian, colorectal, gastric and uterine serous papillary cancers	[146]
KLK11	IGFBPs	Diagnostic and favorable prognostic marker for ovarian and prostate cancers Diagnostic and unfavorable prognostic marker for lung cancer	[147,148]
KLK12		Favorable prognostic marker for breast cancer Diagnostic marker for breast and prostate cancers	[149-151]
KLK13	Plasminogen, ECM component	Favorable prognostic marker for breast and ovarian cancers	[152,153]
KLK14	Matrilin, laminin, collagens, ECM component	Diagnostic and unfavorable prognostic marker for breast and prostate cancers Diagnostic and favorable prognostic marker for ovarian cancer	
KLK15	Pro-KLK3, pro-uPA	Diagnostic and favorable prognostic marker for breast cancer Diagnostic and unfavorable prognostic marker for ovarian and prostate cancers	[101,154,155]

ECM: Extracellular matrix; IGFBP: Insulin-like growth factor binding protein; KLK: Tissue kallikrein; LMW: Low molecular weight; MMP: Matrix metalloproteinase; PAP: Prostatic acid phosphatase; pro-uPA: Pro-form of urokinase-type plasminogen activator; VIP: Vasoactive intestinal peptide.

proliferation and invasiveness. As for other type of cancers, there are at least eight genes (*KLK3, KLK5, KLK6, KLK8, KLK10* and *KLK12 – KLK14*), three genes (*KLK5, KLK10* and *KLK14*), two genes (*KLK6* and *KLK10*) and one gene (*KLK11*) of the *KLK* family that have been reported to be dysregulated in breast, testicular, pancreatic and lung cancers, respectively [83-86]. Taking *KLK10* as an example, although no proteolytic activity has yet been detected, it is differentially regulated in endocrine-related tumours and has potential as prognostic and/or diagnostic marker [87]. Its mRNA and protein levels became decreased or absent in several types of cancers, including breast cancer, cervical cancer, prostate cancer and acute lymphocytic leukaemia. On the contrary, transfection of *KLK10* into some of the *KLK10*-negative cancer cells resulted in significant reduction of the tumorigenicity, providing a potential approach to restore *KLK10* expression in patients with these types of cancers [87]. Inexplicably, however, the expression of *KLK10* is found to be upregulated in ovarian, colorectal, gastric and uterine serous papillary cancers. These results point to the paradoxical role of *KLK10* in human cancers and underscore the importance of further studies of this kallikrein gene. Functional characterisation of KLK13 and KLK14, two novel members of the KLK family, revealed that they both possess a trypsin-like specificity and are involved in several facets of tumour progression including growth, ECM degradation, invasion and angiogenesis. These findings may have clinical implications for the management of cancers in which KLK13 or KLK14 activity is elevated [88,89]. Taken together, members of the KLK family have the potential for use as promising RNA and/or serological markers in the early detection of cancer diseases, especially in hormone-dependent malignancies (**Table 1**). At the time of this writing, dozens of patent applications have either been approved (see **Table 1**) or filed (mostly by Bayer Healthcare [129-155]) that cover not only the aforementioned findings, but also the diagnostic and therapeutic usage of different KLKs in cancer diseases.

There is now ample evidence that certain *KLK* splice variants are also differentially expressed in cancer, although their biological significance remains to be elucidated. For instance, the expressions of six *KLK* variants, including three for *KLK4* (an 83-bp insertion of intronic sequence from intron 3, a complete deletion of coding exon 4 with or without an additional 12-bp insertion of intronic sequence from intron 2), one for *KLK5* (with a shorter 5′-UTR region), one for *KLK7* (with a longer 3′-UTR fragment), and two for *KLK8* (missing either coding exon 2 or exon 3) were all shown to be upregulated at relatively high levels in cancerous ovarian tissues and cells. Additionally, *KLK13* has at least five tissue-specific alternative transcripts that are expressed in normal testicular tissues, but absent in the adjacent cancerous tissues [90]. These alternative *KLK* transcripts might herald a new generation of tumour markers. Although it is presently unclear which mechanism(s) lead to differential expression of KLK transcripts, it is believed that epigenetic modifications in tumour cells is a key factor for modulating the expression levels of KLKs, regardless in wild type or variant form.

As mentioned above, the KLK serine proteinase family appears to have a dual role in cancer diseases, in that they are able to either promote or inhibit tumour-cell growth, angiogenesis, invasion and metastasis. For one, the excessive expression and activity of certain KLKs can not only promote tumour growth by increasing the bioavailability of growth factors that reside in the ECM, but can also enhance the degradation of the ECM components to facilitate tumour cell invasion to the lymphatics or blood vessels (**Table 1**). For example, KLK3 and KLK11 are known to play a crucial role in prostate and breast cancers by cleaving insulin-like growth factor binding proteins (IGFBPs), thus liberating insulin-like growth factors IGF1 and IGF2, which are important mitogenic peptides involved in regulating normal and malignant cellular proliferation, differentiation, apoptosis and transformation [21,91,92]. In addition to the cleavage of IGFBPs, KLK3 can stimulate the production of reactive oxygen species (ROS) that subsequently induce apoptosis or lead to the activation of proto-oncogenes and inactivation of tumour-suppressor genes. KLK3 also cleaves and activates transforming growth factor β (TGF-β), thereby stimulating cell detachment to smooth the progress of tumour spread [93]. In pancreatic adenocarcinomas, KLK7 is highly upregulated to increase the cleavage of E-cadherin. The soluble E-cadherin fragments then drastically enhance tumour cell invasion through ECM proteins with a corresponding reduction in cell aggregation [94]. On the contrary, certain KLKs might negatively regulate tumour cell growth. For example, *KLK10* is considered a putative tumour suppressor gene, by virtue of its downregulation in several cancer types [95,96] and because transfection of this proteolytic enzyme into the tumorigenic cancer cell line reduced its anchorage-independent growth. Most recently, a colorimetric ELISA was developed to validate KLK10 serum concentration in normal and breast cancer sera. The result demonstrated that in a subset of breast cancer patients with both early and late stage disease, serum KLK10 levels were elevated, at > 1.55 ng/ml, above all normal female and benign disease samples [97]. Moreover, overexpression of KLK8 was shown to suppress the migratory and invasive abilities of high-invasive lung tumour cells [98]. This phenomenon likely resulted from KLK8 cleaving fibronectin to suppress integrin signalling and retard cancer cell motility [98]. Lastly, several *in vitro* studies have shown that some KLK members, including KLK1 – KLK3, KLK6 and KLK7 can enhance angiogenesis by either catalysing the hydrolysis of ECM proteins to enable both endothelial-cell as well as tumour-cell migration and invasion, or by liberating or activating pro-angiogenic growth factors, such as vascular endothelial growth factor and the activation of pro-MMPs (**Table 1**). However, other KLKs like KLK9 and KLK13 are implicated in the inhibition of angiogenesis, via their release of angiostatin-like fragments from plasminogen to suppress tumour development [99,100]. These studies may provide

explanation on why certain *KLK* genes are biomarkers of favourable prognosis for cancer patients. In conclusion, whether and to what extent the KLK family acts to promote or inhibit neoplastic progression might ultimately depend on the tumour microenvironment and the type of tissues. As the majority of experimental data reviewed in this article are from *in vitro* biochemical and cell culture studies, more direct evidence is needed to determine the roles of KLKs in cancer biology.

6. Expert opinion

Since the first member of the KLK family was identified > 70 years ago, remarkable advances in understanding the catalytic mechanism and functional features of this type of serine proteinases have had important implications for clinical application. To date, KLKs are regarded as major therapeutic targets and points of intervention for a variety of diseases, including hypertension, diabetes and cancer. In the case of cancer, the successful identification and detection of abundant levels of both the wild type and variant KLK mRNAs and proteins in tumour cells as well as in patients' body fluids has led to the utilisation of KLKs as promising RNA and/or serological tumour biomarkers. Approximately dozens of patent applications have either been approved (see **Table 1**) or filed (mostly by Bayer Healthcare) that cover not only the distinct expression patterns, but also the diagnostic and therapeutic usage of different KLKs in various disorders, including cancer. Only the clinical application of *KLK14* has not yet been filed for a patent, indicating that more *in vitro* and *in vivo* works are needed to clarify the physiological role of this novel *KLK* gene. Moreover, as it has recently become practical to combine the diagnostic, prognostic and predictive value of multiple biomarkers into models, the exploitation of new subsets of wild type and/or variant KLKs into a multiparametric panel may provide superior diagnostic/prognostic indications than that of the single KLK marker. Therefore, additional studies are required to evaluate this assumption. By using a positional-scanning combinatorial library of tetrapeptide substrates, the substrate specificities of seven KLK endopeptidases have been recognised. This identification of the specificity profiles of the KLK family members can lead to a better understanding of their enzymatic functions and assist in identifying their physiological protein substrates, as well as in designing more selective inhibitors. It is also important to determine more X-ray structures of either KLK alone or complexed with substrates/inhibitors, as the information shall provide more detailed insights on how KLK enzymes interact with different cognate proteins, thereby making the rational design of low-molecular weight synthetic inhibitors feasible for anticancer drug development and treatment. Few irreversible and reversible inhibitors of different KLKs have recently been made and patented. However, the limiting step in using these synthetic compounds or peptide derivatives in anticancer therapy is not dependent on the identification of an effective inhibitor, but rather relies on the elucidation of the pathological aspects of KLKs in tumour-cell growth, angiogenesis, invasion and metastasis. In addition, members of the KLK family may represent fine targets for immunotherapy because anti-KLK antibodies, such as those of KLK4, were only present in the serum of males with prostate cancer. With this regard, the invention of new KLK inhibitors and antibodies with consistent quality, potency and stability will be the top priority for anticancer therapies. Other important goals for the future work on KLKs also include the identification of their physiological substrates, the evaluation of their biological activity in various signalling pathways, the modes of their regulation and the clinical relevance of different KLK alternative transcripts and proteins during neoplastic progression. Finally, systematic studies in animal models and clinical trials are also required to determine the use of the KLK serine proteinases in the diagnosis, prognosis and treatment of cancer.

Declaration of interest

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202. http://www.gene.ucl.ac.uk/nomenclature
Each human gene is given a unique approved symbol and stored in the HGNC (human gene nomenclature committee) database to facilitate the search and retrieval of information.

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