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# Cellular Proteases as Cancer Biomarkers: A Review

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## Abstract

Over the past few decades a variety of biomolecules have been proposed as diagnostic biomarkers and predictors of severity for transmissible and nontransmissible diseases. Studies in a range of cancers have revealed many biomarkers with great potential in cancer diagnosis, in establishing tumor stage, progression, and response to therapies; such as the Kallikrein and Metalloproteinase families. Traditionally blood (serum) and tissue have been the main biological sources of biomarker discovery, but in the past decade urine has emerged as a promising source of cancer biomarkers. In this review we will focus on two large families, the Kallikrein family of serine proteases discovered in serum, and the Metalloproteinase family of zinc proteases discovered in urine, as potential cancer biomarkers.

## Keywords

Cancer biomarkers, gene, kallikreins, metalloproteinases, protein.

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## Resumen

Durante las últimas décadas una gran variedad de biomoléculas han sido propuestas como biomarcadores de diagnóstico y de predicción de severidad de enfermedades transmisibles y no transmisibles. Estudios realizados en variedad de cánceres han revelado muchos biomarcadores, como las familias Kalikreina y Metaloproteinasas, con gran potencial en el diagnóstico de cáncer, en el establecimiento del grado de cáncer, su progresión y su respuesta a las terapias. Tradicionalmente la sangre (suero) y tejido han sido las principales fuentes biológicas para el descubrimiento de biomarcadores, pero en la última década ha surgido la orina como fuente prometedora de biomarcadores de cáncer. Esta revisión se centra en dos grandes familias, la familia Kalikreina de las serina proteasas, descubiertas en suero, y la familia Metaloproteinasas de las zinc proteasas, descubiertas en la orina; las cuales son biomarcadores potenciales contra el cáncer.

## Palabras clave

Biomarcadores de cáncer, gen, kalikreinas, metaloproteinasas, proteína.

## 1. INTRODUCTION

Biomarkers are molecules whose levels of expression or modifications in the body are indicative of a biological state. These biomarkers can be genetic, epigenetic, proteomic or glycomic, and have great potential in cancer diagnosis, in establishing tumor stage, progression, and response to therapies. Improved understanding of cancer pathogenesis and the development of advanced techniques has revealed a large number of potential tumor markers, such as the Kallikrein and Metalloproteinase families.

DNA, RNA and protein biomarkers are the most commonly studied. Traditionally, blood has been used as the primary biological sample for the discovery of biomarkers (Omenn, 2004); its biggest advantage being that it is in contact with all the cells in the body. However, using blood for proteomic analysis also has several disadvantages, such as the inevitable activation of proteases during sample collection, which generate an array of proteolytic products and thus introduce variability to the sample (Omenn et al., 2005). Additionally, blood has 20 high abundance proteins corresponding to 99% of the total protein (Veenstra et al., 2005), which mask the other less abundant proteins during proteomic analysis. Due to these issues urine has emerged as a potential source of biomarkers. The Human Proteome Organization (HUPO) announced in October 2005 the inclusion of the Human Kidney and Urine Proteome Project (http://hkupp.kir.jp/) as one of their projects sanctioned under the Disease Biomarker Initiative (DBI). In contrast to blood, urine collection is minimally invasive, sample handling is simple and it has been shown that urine samples are particularly stable (Schaub, et al., 2004; Theodorescu et al., 2006) which significantly reduces variability. The main disadvantage of urine is the variation in protein concentration due to differences in fluid intake during the day, but this is controlled through protein standardization to creatinine in the sample (Vestergaard & Leverett, 1958).

Thus, along with blood, urine has emerged as a promising biological source not only for the discovery of biomarkers of

diseases of the genitourinary tract (urological cancers, prostate cancer, etc.), but also of diseases affecting other organs of the body (brain tumors, breast cancer, and others). The first study on biomarkers in urine identified markers of brain tumors, breast, ovarian and bladder cancer (Moses et al., 1998). Most of these biomarkers were angiogenic factors (promote the formation of new blood vessels) belonging to the matrix metalloproteinase family (MMP).

Another protease family which is found in serum of cancer patients and widely researched is the Kallikrein family. Initially it was thought there were only 3 members in this family (Riegman et al., 1992), but from 1995 onwards extensive work by several research groups has revealed a total of 15 members in the human kallikrein gene family. In this review we will focus on these two protease families, the kallikrein family of serine proteases found in blood, and the metalloproteinase family of zinc proteases found in urine; both potential biomarkers in cancer diagnosis and progression.

## 2. KALLIKREIN FAMILY: SERINE PROTEASES

### 2.1 Characteristics

The kallikrein family is a group of proteins with enzymatic activity. Initially, only three kallikrein proteins were identified (classic hK): pancreatic-renal kallikrein (hK1) was first characterized in the 1930s, followed by prostate kallikrein (hK3) which was discovered from several independent studies in the 60s (Yousef & Diamandis, 2001). The Kallikrein family was finally defined as such due to the subsequent characterization of a protein called human glandular kallikrein (hK2) (Enami & Diamandis, 2008). Studies in the 90s, led to the identification of new family members, due to the high homology observed with the classical kallikreins at both the genomic and structural level (Yousef & Diamandis, 2001). There are currently 15 proteins with serine-protease activity in this family, mentioned in Table 1.

Table 1. Kallikrein Family Members

| Gene/Protein | Disease | References |
|---|---|---|
| *KLK1* / hK1 | Sepsis, pancreatic disease, cancer | Yousef & Diamandis, 2001 |
| *KLK2* / hK2 | Breast and prostate cancer | Partin et al., 1999 |
| *KLK3* / hK3 | Breast and prostate cancer | Poliouras & Diamandis, 2006 |
| *KLK4* / hK4 | Ovarian cancer | Prezas et al., 2006 |
| *KLK5* / hK5 | Ovarian cancer | Dong et al., 2003 |
| *KLK6* / hK6 | Ovarian and breast cancer, Alzheimer's disease | Pampalakis & Sotiropoulou, 2006 |
| *KLK7* / hK7 | Ovarian cancer, psoriasis, keratinization | Dong et al., 2003 |
| *KLK8* / hK8 | Ovarian cancer, Kindling epilepsy | Magklara et al., 2001 |
| *KLK9* / hK9 | Ovarian cancer | Yousef et al., 2001 |
| *KLK10* / hK10 | Breast and prostate cancer | Sidiropoulos et al., 2005 |
| *KLK11* / hK11 | Ovarian and prostate cancer | Luo et al., 2006 |
| *KLK12* / hK12 | Breast cancer | Borgoño et al., 2004 |
| *KLK13* / hK13 | Breast cancer | Chang et al., 2001 |
| *KLK14* / hK14 | Breast cancer | Poliouras & Diamandis, 2006 |
| *KLK15* / hK15 | Prostate cancer | Borgoño et al., 2004 |

The *KLK* genes are flanked by noncoding regions at both the 5' and 3' ends (NTR5' and NTR3', Non-translated region) (Fig. 1). There can be two NTR regions at the 5' end, whereas only one NTR has been characterized in the 3' end (Kurlender et al., 2004). These genes contain five exons (designated from 1 to 5), which are highly conserved in all 15 KLK family members (Fig. 1). A pattern

of alternating intron regions of variable size, designated 0, I, II and III, can be observed between exons (Yousef & Diamandis, 2001). Generally, the initiation codon (AUG) is located 8-87 base pairs downstream of exon 1, while the stop codon is located between nucleotides 150-189 after the start of the coding segment of exon 5 (Pampalakis & Sotiropoulou, 2007).

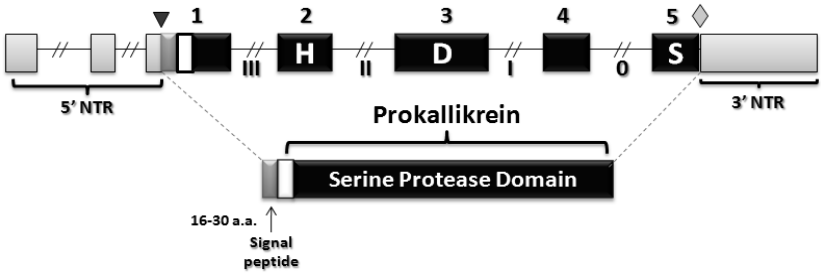


Fig. 1. Structural features of a conserved Kallikrein gene H, D and S correspond to the catalytic histidine, aspartic acid and serine residues located in the active site. Black boxes (1 to 5) indicate coding sequences. Gray boxes represent introns (0 to III). Kallikrein genes are flanked by non-coding regions at 5' and 3' termini, ▼Start codon. ◇ Stop codon.

hK proteins synthesized from *KLK* genes, are single-chain peptides secreted as preproenzymes. As shown in Fig. 1, each hK contains a 16-30 amino acid signal peptide in the N-terminus, followed by a 4-9 amino acid pro-peptide and a catalytic domain that is activated by successive post-translational cleavages during its maturation stage in the secretory pathway (Yousef & Diamandis, 2001). Generally, mature and catalytically active hK proteins are 227-252 amino acids long with approximately 10 to 12 cysteine residues which are important in the formation of disulfide bonds, and consequently for the tertiary structure; and have a highly conserved catalytic triad containing histidine (H), aspartic acid (D) and serine (S) residues (Gomis-Ruth et al., 2002).

## 2.2 Utility of Kallikreins in Biomedical Research

Biochemical studies have determined that kallikreins are differentially expressed in human tissues, fluids and organs,

enabling their use as biomarkers (Yousef & Diamandis, 2001). Differential transcription of messenger RNA (mRNA) and/or protein expression has been observed in: central nervous system, thyroid, prostate, seminal fluid, testis, ovary, uterus, colon, skin, heart, breast, lung and trachea (Shaw & Diamandis, 2007), and changes in these kallikreins have been described in several pathological processes such as inflammation, hypertension, kidney disease, pancreatitis and cancer, among others (Table 1).

### *2.2.1 Prostate cancer biomarkers*

Prostate cancer is the second most common cancer in Colombian men, with an estimated annual mortality rate of 2.885 men (According to the GLOBOCAN 2002 database). Currently, diagnosis is based on serum measurements of prostate specific antigen (PSA, hK3), and a digital rectal examination of the prostate.

hK3, more commonly known as PSA, functions normally within the prostate to liquefy seminal coagulum, however it tends to be upregulated in prostate cancer. Although hK3 concentration is generally higher in serum of patients with prostate cancer, its expression is negatively regulated within the tumor tissue, which is associated with a more aggressive diagnosis (Magklara et al., 2000); hence hK3 has been associated with tumor suppressor activity, pro-apoptosis and negative regulation of cell growth (Diamandis, 2000). hK3 serves not only as a biomarker in cancer diagnosis, but also as a predictor of severity and progression.

Protein hK2 is similar to hK3 (PSA). According to some immunohistochemical studies, hK2 is expressed in higher levels in prostate tumor tissue than in hyperplastic prostate tissue or normal tissue, however other quantitative studies have reported a similar expression trend to hK3 (Finlay et al., 1998; Magklara et al., 2000). In addition to *KLK2* and *KLK3*, other kallikrein family members, such as *KLK5, 6, 10* and *13,* are found to be negatively regulated at the transcriptional level in tumor tissue of patients with prostate cancer when compared with surrounding normal tissue (Hakalahti et al., 1993; Petraki et al., 2003; Sotiropoulou et al., 2003).

### 2.2.2 Endocrine cancer biomarkers

Serine protease hK5 is secreted mainly by skin, testes, brain and mammary gland tissue and presents a high proteolytic activity (Shaw & Diamandis, 2007). Transcription of *KLK5* mRNA is positively regulated by steroid hormones; this has been verified in a human breast tumor cell line (BT-474) and has led to the exploration of mRNA over-expression in endocrine malignancies, revealing an increase of this biomarker in breast, testicular and ovarian cancers (Yousef et al., 2004).

In general, upregulation of mRNA or protein has been observed in kallikreins 4, 5, 6, 7, 8, 10, 11, 12, 13, 14 and 15, both in tissue and serum of patients with ovarian cancer (Table 1). In contrast, mRNA synthesis of kallikreins 3, 10, 12, 13 and 14, was found to be reduced in breast tumor tissue and cell lines derived from breast cancer (Yousef & Diamandis 1999; 2001; Dhar et al., 2001); although *KLK6* mRNA is upregulated in primary breast cancer tissue, it is transcribed at a reduced rate in metastatic breast cancer (Anisowicz et al., 1996). In particular, transcription of *KLK5* and *KLK14* is decreased in breast cancer, although in some patients it is possible to detect elevated levels of proteins hK5 and hK14 in serum (Borgoño et al., 2006). This increased rate of hK synthesis can be explained by glandular destruction processes or angiogenesis, which are common events in the development of neuroendocrine tumors and may increase the level of hK proteins in the intravascular compartment.

Increased mRNA levels of *KLK5, KLK10* and *KLK14* have been reported in patients with testicular cancer when compared to healthy individuals (Luo et al., 2003), and transient transfection of the *KLK10* gene in breast cancer derived cell lines decreased the anchorage independent growth of these cells and their ability to form tumors (Roman-Gomez et al., 2004). These kinds of results have led to the conclusion that *KLK10* is a tumor suppressor gene.

These kallikrein biomarkers are not only useful in cancer diagnosis, but are also serological markers of prognosis. For example, finding high mRNA or protein levels of kallikreins 4, 5, 6, 7, 10 and 15 is considered a poor prognosis for the patient, given

that studies have shown a more severe clinical outcome and lower rates of survival in these patients (Preza et al., 2006; Enami & Diamandis 2008). By contrast, kallikreins 8, 9, 11 and 14 are markers of good prognosis; high concentrations of these markers are associated with higher rates of survival and recovery from illness (Borgoño et al., 2004).

## 3. METALLOPROTEINASES: ZINC PROTEASES

### 3.1 Metalloproteinase Superfamily

The metalloproteinase superfamily encodes a highly conserved zinc-binding motif containing three histidine residues which bind zinc in the active site. This superfamily includes matrix metalloproteinases (MMPs) and ADAMs (a disintegrin and metalloproteinase) (Stöcker et al. 1995).

The MMP family has 28 closely conserved members. Most MMPs share a common structure, composed of a pro-peptide domain, a catalytic domain, a hinge region, and a hemopexin-like domain (Fig. 2). At least 15 members are Zn-dependent; their activity relies on a $Zn^{2+}$ metal ion bound within the catalytic domain in the conserved HExxHxxGxxH sequence (Fig. 2). The pro-peptide domain can bind to the Zn ion in the catalytic domain keeping the enzyme inactive, while the hemopexin-like domain is thought to be involved in protein-protein interactions, which helps determine substrate specificity. MMPs are synthesized as pro-enzymes and most are secreted before being converted to their active form; in order to be activated the pro-domain must be cleaved from the catalytic domain. A signal peptide at the N-terminus targets these proteins for the secretory pathway and is removed prior to secretion from the cell. Although most MMPs are secreted proteins, integral membrane MMPs have also been described (MT-MMPs), anchored to the cell membrane by a transmembrane and intracytoplasmic domain (Jones et al., 2003).

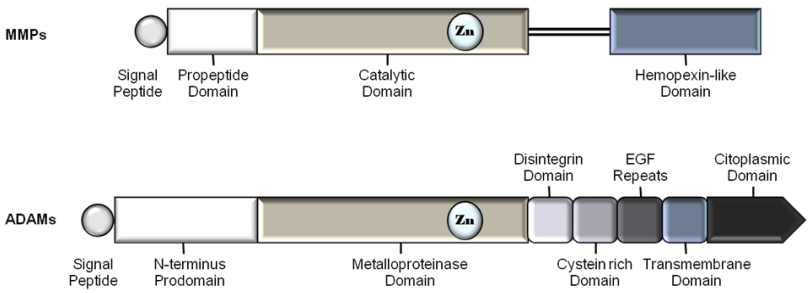


Fig. 2. Structural domains of the MMP and ADAM families. The structures shown in this figure describe the majority of MMP and ADAM family members

The main role of these human zinc proteases is in tissue remodeling. This function is exploited by tumor cells which use MMPs to break down components of the extracellular matrix, thus allowing malignant cell migration to other parts of the body (metastasis) (Fig. 3). Leukocytes that are involved in inflammatory processes are the main source of MMPs (Fig. 3).

The ADAM family (a disintegrin and metalloproteinase) is closely related to the MMP family as they are also zinc metalloproteinases and have an extracellular metalloproteinase domain (Fig. 2). ADAM proteins possess a prodomain, metalloproteinase, disintegrin, cysteine rich, EGF, transmembrane and cytoplasmic domains (Fig. 2). The metalloproteinase domain is similar to that found in MMPs and contains the same zinc-binding site. The disintegrin domain binds integrin and inhibits platelet aggregation. The cystein rich and EGF domain have many cystein residues which form disulfide bonds; however, the function of these domains is not yet well understood. Most ADAMs are integral membrane proteins, anchored with a C-terminus transmembrane domain and containing a cytosolic domain which is involved in signalling; but some members of the ADAM family are cleaved and targeted to the secretory pathway.

These proteins play a role in cell-cell and cell-matrix interactions, and in recent years evidence has emerged of ADAM proteins in tumor tissues, acting specifically in angiogenesis and metastasis processes (Lu et al., 2008) (Fig. 3).

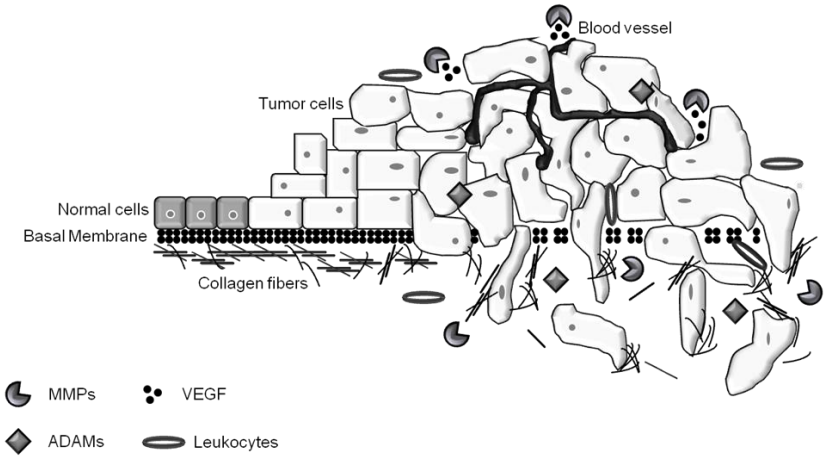


Fig. 3. Role of MMPs and ADAMs in cancer pathogenesis. MMPs and ADAMs modulate the tumor microenvironment by increasing the availability of pro-angiogenic factors like VEGF, thus promoting the formation of new blood vessels that feed the tumor. They also degrade the components of the basal membrane and extracellular matrix, facilitating the migration of cancer cells to other parts of the body

## 3.2 Utility of MMPs in Biomedical Research

### *3.2.1 Brain tumor biomarkers*

The incidence of metastatic brain tumors is on the rise (Barker et al., 2005). The main problem in treating brain tumors is the lack of diagnostic tools to detect this disease. Two contemporary studies in 1998 addressed this issue by reporting the presence of enzymes in brain tumors (Surawicz et al., 1998) and in urine samples taken of these patients (Moses et al., 1998). Based on these publications a more detailed study was initiated in 2008 to examine the effectiveness of these proteins as potential noninvasive biomarkers of brain tumors (Smith et al., 2008).

A study with patients with a variety of brain tumors led to the discovery of metalloproteinases MMP-2, MMP-9, MMP-9/NGAL and VEGF (vascular endothelial growth factor) in urine samples collected at random, whereas these were not found in urine samples of the healthy control group (Smith et al., 2008). The

expression patterns of these molecules found in urine reflect the expression patterns in brain tissue; patients with decreased MMP-2 activity in urine also had low MMP-2 levels of expression in the tumor tissue, while patients with high MMP-9 activity in urine retained elevated levels of MMP-9 in the tumor. Additionally it was shown that elevated levels of these biomarkers in urine decreased after effective surgical treatment of the tumor. It is important to note that in serum elevated levels of MMP-9 have also been detected, in patients with colorectal cancer (Wilson et al., 2006), breast cancer (Quaranta et al., 2007) and ovarian cancer (Lin et al. , 2009).

As shown in Fig. 3, this family of metalloproteinases modulate the tumor microenvironment in several ways: a) Encouraging tumor growth by releasing insulin-like growth factor IGF. b) Promoting the formation of new blood vessels (angiogenesis) that feed the tumor, by increasing the availability of pro-angiogenic factors like VEGF. c) Facilitating the migration of cancer cells (metastasis) by degrading the extracellular matrix components. In particular two members of this family, MMP-2 and MMP-9, degrade collagen, fibronectin and laminin, which are major components of the basal membrane.

### *3.2.2 Brest cancer biomarkers*

Breast cancer remains the most common cancer among women and the second leading cause of death by cancer (Parkin & Fernandez, 2006). The priority is to identify women at high risk of developing breast cancer.

Recently, interest in a protein called ADAM-12 as a potential biomarker of breast cancer has increased. Immunohistochemical studies of breast tumor tissue have demonstrated increased expression of ADAM-12 and MMPs (Iba et al., 1999), which have also been found in high levels in the urine of these patients ($P<0.001$) (Moses et al., 1998). A well-designed study showed that women with high levels of MMP-9 and ADAM-12 in urine are 5 times more likely to develop atypical hyperplasia and 13 times more likely to develop lobular carcinoma in situ (Pories et al., 2008), which are precursors of cancer. So, these biomarkers not

only act as indicators of the presence of breast cancer, but also as high risk predictors. It is reported that ADAM-12, expressed by cancer cells, accelerates breast cancer progression by inducing apoptosis of stromal cells (Kveiborg et al., 2005) and by degrading several extracellular matrix components such as collagen type IV and fibronectin (Roy et al., 2004).

## 4. CONCLUSIONS

The effectiveness of the Kallikrein and Metalloproteinase families as tools in cancer diagnosis, in monitoring progression, therapeutic success and recurrence of the disease, has been demonstrated. As can be seen from many of these studies, urine has emerged as an ideal non-invasive source of biomarkers; although it is a relatively recent area of work, it is one which is receiving much attention.

In the future it will be necessary to develop panels of biomarkers, given that in many cases a diseased state cannot be effectively defined using a single biomarker. For example, high levels of MMP-9 were detected in the urine of patients with brain tumors, breast and prostate cancer, among others, so MMP-9 could be used as an indicator of the existence of cancer, but would not indicate the type of cancer. A combination of general cancer biomarkers and specific biomarkers would be an optimal detection panel.

To date, of the biomarkers mentioned in this review, only hK3 (PSA) has been clinically implemented as a biomarker (of prostate cancer). Along with the discovery of new biomarkers, the challenge now will be the validation of the existent biomarkers in a large study population, and their clinical implementation.

## 5. ACKNOWLEDGEMENT

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