REVIEW

Molecular and pathobiological insights of bikunin/UTI in cancer

Antonio Junior Lepedda[1] ⬤ · Gabriele Nieddu[1] ⬤ · Claudia Cannas[1] ⬤ · Marilena Formato[1] ⬤

Abstract

Bikunin is a small chondroitin sulfate proteoglycan (PG) with Ser-protease inhibitory activity that plays pleiotropic roles in health and disease. It is involved in several physiological processes including stabilization of the extracellular matrix (ECM) of connective tissues and key reproductive events. Bikunin is also implicated in both acute and chronic inflammatory conditions and represents a non-invasive circulating and/or urinary (as Urinary Trypsin Inhibitor or UTI) biomarker. It exerts inhibitory effects on urokinase-type plasminogen activator (uPA) and its receptor (uPAR) mediating tumor invasiveness by a down-regulation of uPA mRNA expression, thus representing an anti-metastatic agent. However, only limited data on its potential as a diagnostic and/or prognostic marker of cancer have been reported so far. Recent technological advances in mass spectrometry-based proteomics have provided researchers with a huge amount of information allowing for large-scale surveys of the cancer proteome. To address such issues, we analyzed bikunin expression data across several types of tumors, by using UALCAN proteogenomic analysis portal. In this article we critically review the roles of bikunin in human pathobiology, with a special focus on its inhibitory effects and mechanisms in cancer aggressiveness as well as its significance as cancer circulating biomarker.

Introduction

Cancer is an ever-growing healthcare problem, being the second leading cause of death globally, with an estimated 9.6 million deaths in 2018 (https://www.who.int/health-topics/cancer). It is a group of related diseases caused by genetic mutations leading to abnormal cell proliferation and spreading throughout the body.

Tumor invasion is a hallmark of human cancer and is the major contributor to the morbidity and mortality of the disease. Although many signs of progress have been done in developing therapeutic strategies against primary tumors, targeting tumor metastasis has achieved only limited success. This process involves a deep remodeling of the pre-existing ECM, which physiologically provides cells with chemical signals and mechanical support essential for maintaining tissue homeostasis [1], allowing to support the

continued expansion of the tumor mass and the metastatic process [2]. Among the several matrix-degrading enzymes implicated in this process there are proteases such as matrix metalloproteinases (MMPs), plasminogen activators, cathepsins, as well as glycosidases such as heparanase and hyaluronidases [3]. In this respect, a large body of evidence shows that the urokinase-type plasminogen activator and its receptor (uPA/uPAR system) play key roles in mediating proteolysis during cancer invasion and metastasis and correlate with both tumor invasiveness and poor prognosis [4]. Indeed, the serine protease uPA converts plasminogen into plasmin, another serine protease that, in turn, may degrade several components of both the basement membrane and the ECM, including laminin, vitronectin, and fibronectin, therefore, facilitating the migration of tumor cells through the ECM and basement membrane barriers. Expression of uPA and uPAR can be upregulated by mitogens, growth factors, oncogenes, and binding of integrin with ECM proteins [5]. Hence, the uPA/uPAR system may represent a potentially relevant therapeutic target [4].

Bikunin is a small circulating proteoglycan (PG) found also in urine, as Urinary Trypsin Inhibitor (UTI) or

✉ Antonio Junior Lepedda
ajlepedda@uniss.it

1   Department of Biomedical Sciences, University of Sassari, Sassari, Italy

ulinastatin, and in amniotic fluid, with inhibitory activity against serine proteases. Although bikunin cannot inhibit directly uPA activity, it exerts a down-regulation of uPA mRNA expression and protein secretion leading to an inhibition of both in vitro and in vivo tumor cell invasion and metastasis [6–9]. In this article, we critically review the roles of bikunin in human pathobiology, with a particular emphasis on cancer propagation. Furthermore, its significance as a potential diagnostic and/or prognostic biomarker in various cancer types will be discussed, also in light of the new information obtained by analyzing the most recent cancer proteogenomics databases.

Search strategy and data analysis

A comprehensive literature search of the Medline/PubMed database was performed on the following topics: bikunin/urinary trypsin inhibitor structure and metabolism, roles in different pathophysiological conditions with a particular emphasis on tumors cells invasion through the uPA/uPAR system, usefulness as tissue and circulating biomarker. This manuscript reviews the major literature on this research field since 1995 and shows an overview of the main findings. Furthermore, it provides insight into the potential of bikunin as a cancer biomarker by analyzing the most recent high-throughput gene- and protein-expression databases, performed with the search engine UALCAN (The **U**niversity of **AL**abama at Birmingham **CAN**cer data analysis Portal). UALCAN is an interactive web resource (http://ualcan.path.uab.edu/index.html) that allows for the analysis of publicly available cancer OMICS data. Among the possible applications, it enables the identification of biomarkers or the in silico validation of potential genes of interest. Furthermore, it provides graphs and plots depicting expression profile and patient's survival information. The available databases have been provided by The Clinical Proteomic Tumor Analysis Consortium (CPTAC) (https://gdc.cancer.gov/about-gdc/contributed-genomic-data-cancer-research/clinical-proteomic-tumor-analysis-consortium-cptac), and by The Cancer Genome Atlas (TCGA) consortium (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). The CPTAC of the National Cancer Institute (NCI) at the National Institutes of Health (NIH) is devoted to accelerating the understanding of the molecular basis of cancer through the application of large-scale proteome and genome analyses, or proteogenomics. TCGA is a landmark cancer genomics program developed by the joint effort between NCI and the National Human Genome Research Institute.

## Bikunin/UTI structure and biosynthesis

Bikunin is a chondroitin sulfate PG (CSPG) with multipotent inhibitory effect on several Ser proteinases, including trypsin, chymotrypsin, granulocyte elastase, kallikrein, cathepsin G, acrosin, and plasmin [10]. It carries both an O-linked low-charge CS chain, at Ser^{10}, and a N-linked oligosaccharide, at Asn^{45} [10–12]. Its protein moiety consists of 147 amino acid residues folded in two Kunitz-type domains (from residues 22 to 77 and from 78 to 133, respectively), containing three disulfide bonds each, as well as N- and C-terminal sequences of 10–25 amino acid residues [11] (Fig. 1).

The whole CSPG has a molecular mass of about 25–26 kDa, being composed of a 16 kDa polypeptide chain, a 2 kDa N-linked oligosaccharide and a 7 kDa CS chain. This latter is constituted of 12–18 disaccharide repeating units, consisting of glucuronic acid (GlcA) and N-acetyl galactosamine (GalNAc), connected to Ser^{10} via a linkage tetrasaccharide (Xyl-Gal-Gal-GlcA). About 25% of GalNAc residues are sulfated at C-4, frequently near the reducing end of CS chain [13–15] (Fig. 1).

Bikunin is synthesized primarily by hepatocytes as a precursor of 352 amino acids (AMBP protein), containing also alpha-1-microglobulin, encoded by Alpha-1-Microglobulin/Bikunin Precursor (AMBP) gene (located on chromosome 9q32), which consists of 10 exons and 9 introns of about 18 kb length, overall (https://www.ncbi.nlm.nih.gov/gene/259) [16]. During maturation, the precursor is proteolytically cleaved into alpha-1-microglobulin (residues from 1 to 205) and bikunin (residues from 206 to 352) [17]. Following glycosidic modification in the Golgi apparatus, bikunin is covalently linked to the C-terminal amino acid residue of one or two polypeptides, called the heavy chains (HCs), through an ester bond with a non-sulfated GalNAc residue of the CS chain, and is released in plasma almost exclusively as a subunit of inter alpha inhibitor (IαI) family molecules [18–20].

In addition to liver parenchyma, which is the main site of AMBP protein synthesis, this gene is expressed at low levels in many tissues/organs (https://gtexportal.org/home/gene/ENSG00000106927#eQTLBlock; https://www.proteomicsdb.org/proteomicsdb/#protein/proteinDetails/51583/expression). For its part, bikunin is widely detectable due to both in situ synthesis and adsorption from circulation [21]. Bikunin expression in tissues varies widely also in relation to pathophysiological conditions including cancer, as shown in the last part of this review.

Fig. 1 Representative schematic structure of bikunin/UTI. Amino acid residues are reported as circles, while monosaccharides are drawn according to the Symbol Nomenclature for Glycans (https://www.ncbi.nlm.nih.gov/glycans/snfg.html). Kunitz I and II domains are reported in light blue and green, respectively. The low-sulfated CS chain, composed of 12–18 repeating disaccharide units consisting of glucuronic acid (GlcA) and N-acetyl galactosamine (GalNAc), is bound to Ser^{10}, while a biantennary N-linked oligosaccharide is bound to Ans^{45}. Chemical structures of the CS non-sulfated (left) and monosulfated at C-4 (right) disaccharide units are reported
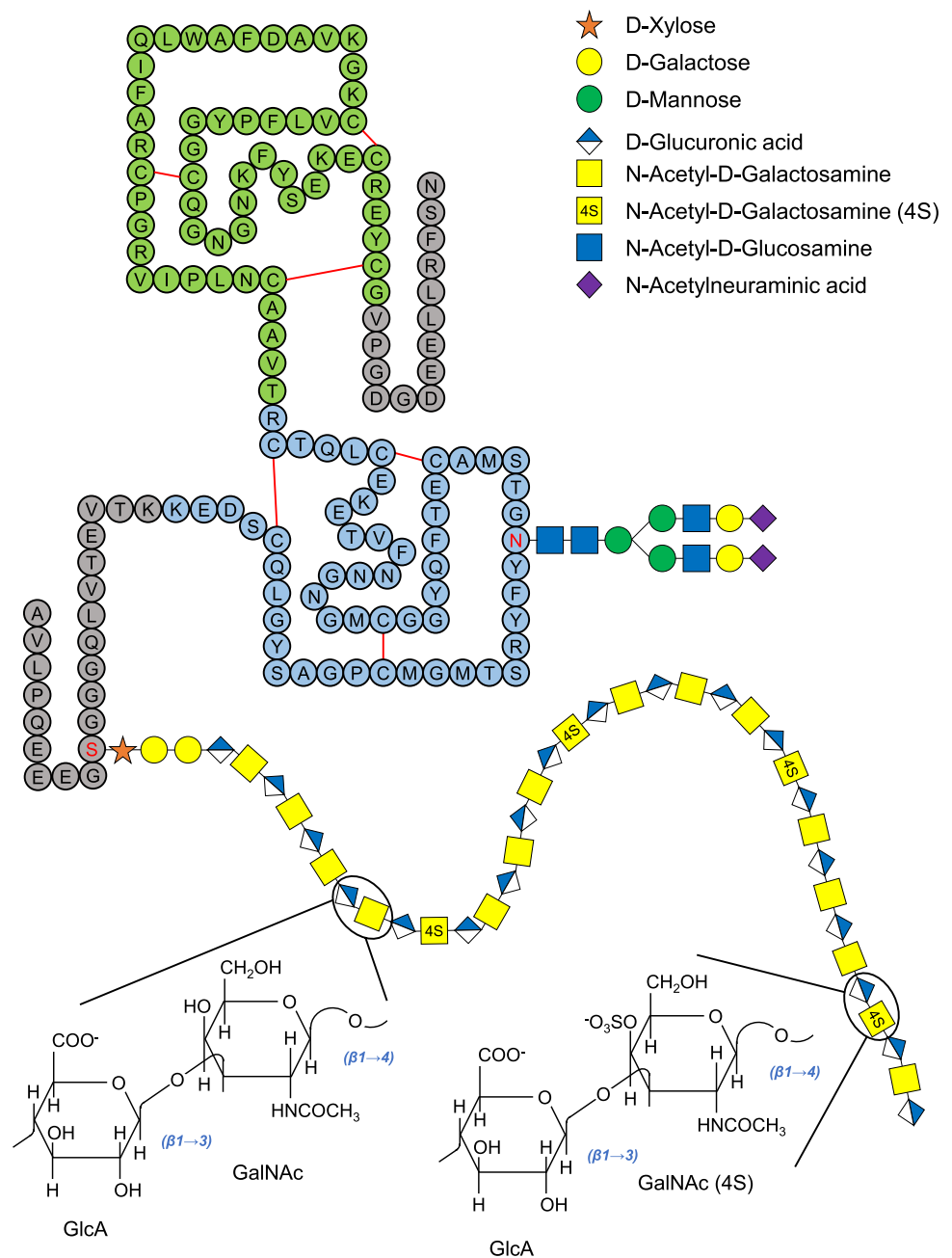
Pathobiological roles of bikunin/UTI

IαI molecules are recruited from circulation to extravascular sites, where the HCs are transferred from the CS chain of bikunin to the locally synthesized hyaluronan (HA), thus forming the serum-derived HA-associated protein-HA complex (SHAP-HA), which supports the formation of new ECM and its stabilization. The formation of this complex has been often associated with inflammatory conditions [19]. Bikunin is essential in these processes because it is required for presenting HCs to HA, as proven by gene knock out studies carried out on murine model [22].

The transfer of HCs from the CS chain of bikunin to the C-6 of the GlcNAc residues of cell-secreted HA, by a transesterification reaction to form the SHAP-HA complex, requires the formation of a covalent intermediate between HC and the transferase tumor necrosis factor stimulated gene-6 protein (TSG-6), followed by the release of bikunin [23–25]. In this process, the bikunin CS chain sulfation pattern has been reported to be crucial for the transesterification of HCs to HA [26].

The release of bikunin from IαI molecules is considered a regulatory mechanism since it leads to the recovery of some bikunin activities that are lost when the complex is

formed. In this context, besides its Ser-proteinase inhibitory activities [27, 28], bikunin is involved in inhibition of lipopolysaccharide-induced lethality [29–31], in smooth muscle contraction [32, 33], in neutrophil release of elastase [34], in mast cell release of histamine [35], in suppression of immune cells [36, 37], and in urolithiasis [38, 39], as well as in stabilization of lysosomal membranes [40, 41], representing a substantial anti-inflammatory agent [42]. Recent studies have shown that bikunin can also play important anti-fibrotic and organ protective roles with important therapeutic implications in chronic kidney disease [43].

Bikunin plays also key roles in different reproductive events, such as cumulus oocyte complex formation, pregnancy, and delivery, beyond being related to the most common pregnancy complications such as pre-term delivery, pre-eclampsia, and gestational diabetes, of which it may represent a non-invasive marker [44]. Because of its proteinase inhibitory activity, bikunin is quickly removed from circulation (approximately 7 min) to prevent a shutdown of repair and healing processes, by both tissue uptake and renal excretion and it is found in urine as UTI or ulinastatin [14]. In human physiological conditions, UTI levels are lower than 5 μg/mL, but they may rise up to tenfold as a consequence of both acute and chronic inflammations including bacterial or viral infections, liver diseases, cancer, type I and II diabetes mellitus, and renal diseases [45–53]. Similarly, modifications of both sulfation degree and chain length of CS moiety have been reported following inflammatory conditions [54]. Hence, bikunin can be considered a positive acute phase protein [42, 54–56].

Bikunin determination is mainly performed by enzyme inhibition assays or immunological detection. The formers allow for a rapid evaluation of the biological activity of bikunin as trypsin inhibitor, based on color shift, with limited specificity and sensitivity. On the other hand, ELISA, immunohistochemistry, and western blotting analyses are all affected by the specificity of the antibody reaction. Furthermore, ELISA cannot discriminate between bikunin/UTI and IαI, whereas, immunohistochemistry and western blotting, due to their intrinsic characteristics, do not allow absolute and reproducible protein quantitation [52].

Bikunin/UTI roles in tumors cell invasiveness

In this section we summarize and discuss on the current knowledge on the inhibitory effects and mechanisms of this small CSPG in cancer invasiveness, using "bikunin" or "UTI", according to the authors that described them.

UTI exerts its inhibitory effects on cell motility and invasiveness by forming membrane complexes with UTI-binding proteins, leading to down-regulation of uPA mRNA expression through negative regulation of PKC- and MEK1/ERK2/c-Jun-dependent signaling pathways [57].

At least two binding sites specific for UTI have been identified on the surface of both neoplastic (human choriocarcinoma SMT-cc1, human chondrosarcoma HCS-2/8, human promyeloid leukemia U937, and murine Lewis lung carcinoma 3LL cells) and normal (human neutrophils, human umbilical vein endothelial cells, fibroblasts, and myometrial cells) cells, a 40-kDa UTI-Binding Protein (UTI-BP40) and a 45-kDa UTI-BP (UTI-BP45) [58]. UTI-BP40 is very similar to a truncated form of human cartilage link protein (LP) and is attached to the cell membrane via a hyaluronic acid anchor [59], whereas UTI-BP45 is a putative CD44 accessory protein [58]. By using truncated forms of UTI (nonglycosylated UTI or carboxyterminal fragments), Hirashima et al. demonstrated that the binding of UTI to UTI-BP45 occurs via the CS moiety [58]. Furthermore, they found that incubation of human chondrosarcoma cell line HCS-2/8 with an anti-LP antibody or soluble chondroitin 4-sulfate, by blocking the binding to either UTI-BP40 or UTI-BP45, independently abrogated suppression by UTI of phorbol ester-induced uPA expression, therefore demonstrating that the concurrent binding to both BPs by UTI is necessary to inhibit uPA expression.

A more in-depth analysis of the mechanisms which regulate UTI binding to cells was performed by Suzuki et al. using UTI derivatives such as the O-glycoside-linked N-terminal glycopeptide, the N-glycoside-linked C-terminal tandem Kunitz domains, CS devoid UTI, asialo UTI, UTI lacking the N-linked oligosaccharide, and the Kunitz domain II of UTI [60]. By this approach, they found that the presence of either the N-terminal moiety (Ala^1 to Lys^{21} residues) containing the chondroitin 4-sulfate side chain or the Kunitz domain I (Lys^{22} to Arg^{77} residues) was necessary to bind cells, whereas the uPA expression was inhibited only by the UTI derivative lacking the N-linked oligosaccharide [60]. Further studies showed that the binding of UTI to its receptor reduces uPA expression by hindering CD44 clustering (activation) and interaction with HA [61].

In ovarian cancer cells, UTI was found effective in inhibiting uPA expression induced by TGF-β1-mediated calcium release, which is a common signaling response in malignant and non-malignant cells treated with a variety of growth factors [7], as well as in inhibiting plasminogen activator inhibitor type-1 (PAI-1) and collagen synthesis through inhibition of TGF-β1 receptor clusterization [62]. Further confirmation of the described bikunin-mediated suppression of cell invasiveness came from transfection studies in which ovarian cancer cells overexpressing bikunin exhibited significantly reduced uPA mRNA levels, inhibition of ERK1/2 phosphorylation, and an overall reduced invasion capacity, whereas proliferation, adhesion, or migration were unchanged [63].

To identify the whole panel of bikunin-regulated genes, high throughput cDNA microarray screening against 3,126 sequence-verified clones was performed on bikunin-treated or bikunin-transfected ovarian cancer cell lines. The 11 bikunin-stimulated genes and 29 bikunin-repressed genes included transcriptional regulators, oncogenes/tumor suppressor genes, signaling molecules, growth/cell cycle, invasion/metastasis, cytokines, apoptosis, ion channels, ECM proteins, as well as some proteases, according to the recognized anti-metastatic effects of bikunin [63].

Studies on breast cancer showed that UTI may inhibit MCF-7 cell line proliferation and the growth of xenograft breast cancer in nude mice by reducing the expression of the receptor 4 for the CXC chemokine and MMP-9 [8]. Furthermore, UTI was shown to inhibit invasion and metastasis in both primary and MDA-MB-231 cells and in a xenograft mouse model [9]. Inhibitory effects on cell proliferation, cell invasion, and migration by UTI administration were also shown in gastric cancer SGC-7901 cells, where a downregulation of uPA expression and activity were evidenced [64]. The in vitro evidence linking UTI to uPA expression and inhibition of tumor cells invasiveness was also corroborated by an immunohistochemical study on 89 ovarian cancer specimens showing a negative correlation between tissue bikunin and uPA levels [65]. Further support for in vitro findings came from bikunin knockout mice that showed an increased prevalence of lung metastasis with respect to Bik$^{+/+}$ mice, which was significantly reduced by administration of exogenous bikunin [66]. The therapeutic potential of bikunin was also evaluated in a phase I trial involving nine patients with advanced uterine cervical carcinoma after definitive treatment, showing that bikunin was well tolerated by oral administration and was effective in suppressing uPA expression in tumor [67].

## Significance of bikunin/UTI as tissue and circulating biomarker of cancer

Beside the plethora of in vitro mechanistic studies dealing with the inhibitory pathways of tumor invasiveness mediated by bikunin, only scanty data on its potential as diagnostic and/or prognostic marker of cancer have been reported so far. Among them, the above-mentioned immunohistochemical work of Tanaka and co-workers [65] showed bikunin as an independent predictor for disease-free survival and overall survival in ovarian cancer. They performed a retrospective study on the expression of bikunin, uPA and CD68 (macrophages) in 89 tumor specimens, evidencing that i) bikunin localized mainly in the cytoplasm of tumor-infiltrating macrophages, ii) its levels were inversely related with those of uPA, iii) low expression of bikunin was inversely related with lymph node metastasis and disseminated

peritoneal metastasis. The 49 patients with low bikunin expression had a 5-year survival rate of 39% compared to the 63% obtained for the 40 patients with high bikunin levels. They concluded the study suggesting that macrophages-derived bikunin might represent an antiinvasive factor, most likely acting through a down-regulation of tumor-associated uPA expression, with prognostic value [65]. Also, bikunin plasma concentrations were found positively associated with improved survival and patient outcomes [47, 68]. In this respect, Matsuzaki et al. determined bikunin levels in plasma samples, by using ELISA, from 200 healthy controls, 200 patients with benign gynecologic diseases, and 327 patients with ovarian cancer, after treatment with chondroitin lyase ABC. Although the enzymatic pre-treatment did not allow to discriminate between the complex and the free form, and any tumor-specific bikunin isoform was evidenced, they were able to identify plasma bikunin as a strong and independent favorable prognostic marker for ovarian cancer.

Recently, Sekikawa et al., by immunohistochemical analysis on 95 oral squamous cell carcinoma specimens, observed the underexpression of AMBP precursor (using a monoclonal antibody raised against a full-length recombinant AMBP) significantly associated with a high metastatic potential to cervical lymph nodes and a poor overall survival [69].

In the last years, the application of the recently developed high-throughput technologies such as next-generation sequencing, microarrays, and mass spectrometry-based proteomics has provided a huge amount of information in many research fields including cancer, frequently freely accessible to the research community. Contextually, several web-tools have been developed to perform in-depth analyses of these data. Among them, the interactive web-portal UALCAN has been proven to be valuable in identifying candidate biomarkers for specific cancers, with diagnostic, prognostic, or therapeutic implications [70, 71]. This resource allows for the analysis of high-throughput gene- and protein-expression data obtained by the CPTAC [72, 73] and TCGA consortium [74]. With the aim of providing new information to the poor literature on the potential of bikunin as tissue and circulating biomarker of cancer, we searched through the most recent cancer proteogenomics databases with the in silico platform UALCAN for evaluating bikunin protein expression (Fig. 2) across tumor and normal samples to be compared with its transcript levels (Fig. 3, Table 1), using AMBP as query gene.

Protein expression data on 1152 samples across ten types of cancer (breast cancer, colon cancer, ovarian cancer, clear cell renal cell carcinoma, uterine corpus endometrial carcinoma, lung adenocarcinoma, pancreatic adenocarcinoma, head and neck squamous carcinoma, glioblastoma multiforme, hepatocellular carcinoma) and 689 controls were retrieved. Differential analysis showed a significant
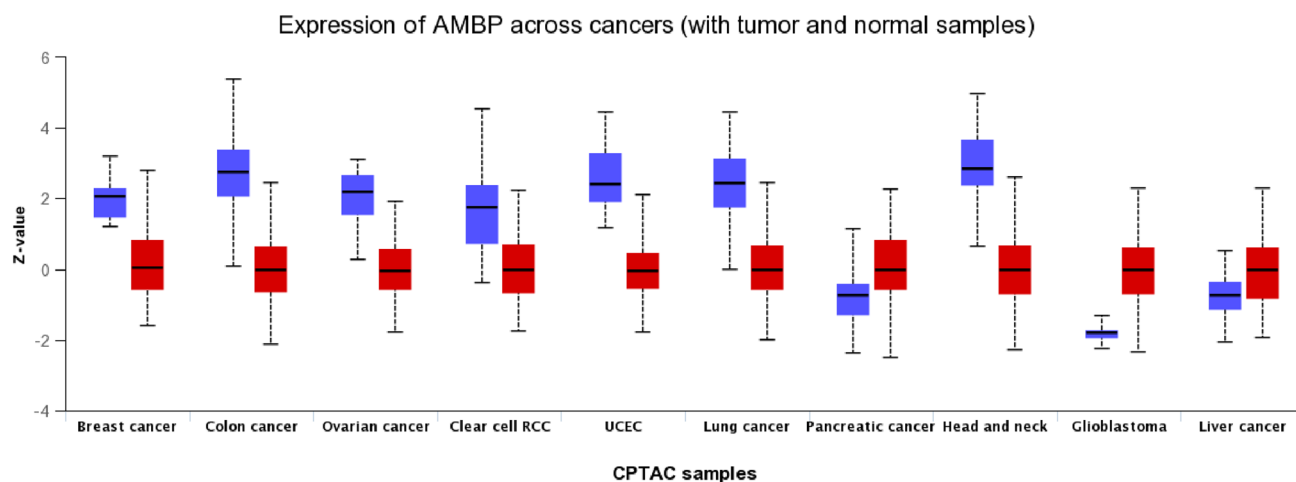
Fig. 2 Box-Whisker plot showing AMBP proteomic expression profile across cancers (blue, normal; red, tumor) obtained by using the web resource UALCAN (http://ualcan.path.uab.edu/cgi-bin/Pan-cancer-CPTAC.pl?genenam=AMBP). UALCAN provides protein expression analysis option using data from Clinical Proteomic Tumor Analysis Consortium (CPTAC) and the International Cancer Prote-ogenome Consortium (ICPC) datasets [70, 71]. Z-values represent standard deviations from the median across samples for the given cancer type. Log$_2$ Spectral count ratio values from CPTAC were first normalized within each sample profile, then normalized across samples. *UCEC* Uterine corpus endometrial carcinoma, *RCC* renal cell carcinoma
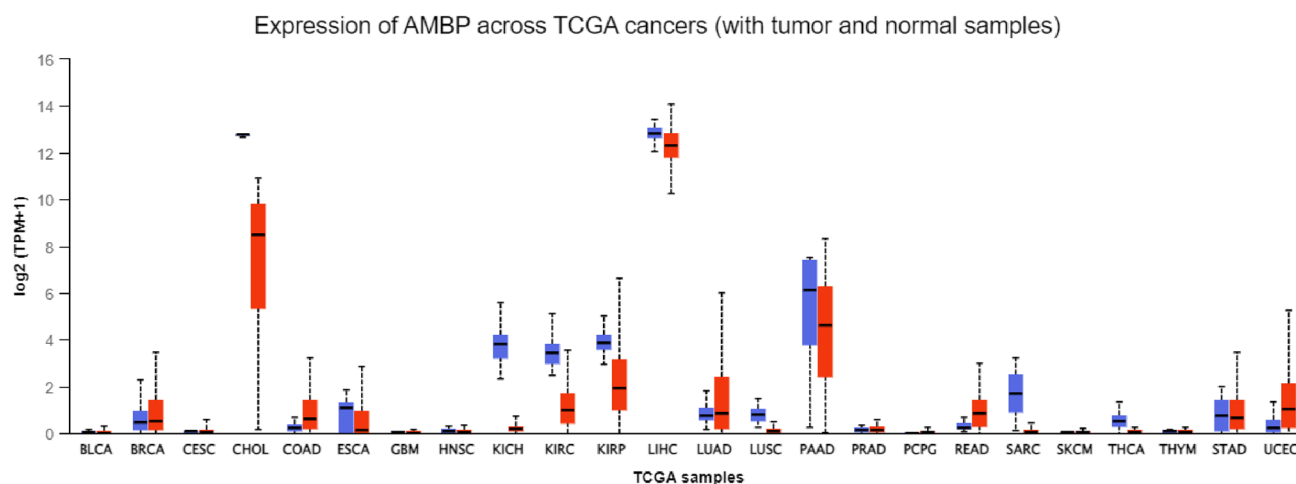
Fig. 3 Box-Whisker plot showing AMBP expression across cancers (blue, normal; red, tumor) included in The Cancer Genome Atlas (TCGA) program, obtained by using the web resource UALCAN (http://ualcan.path.uab.edu/cgi-bin/Pan-cancer.pl?genenam=AMBP). *TPM* transcript per million, *BLCA* Bladder urothelial carcinoma; BRCA: Breast invasive carcinoma, *CESC* Cervical squamous cell carcinoma, *CHOL* Cholangiocarcinoma, *COAD* Colon adenocarci-noma, *ESCA* Esophageal carcinoma, *GBM* Glioblastoma multiforme, *HNSC* Head and Neck squamous cell carcinoma, *KICH* Kidney chro-mophobe, *KIRC* Kidney renal clear cell carcinoma, *KIRP* Kidney renal papillary cell carcinoma, *LIHC* Liver hepatocellular carcinoma, *LUAD* Lung adenocarcinoma, *LUSC* Lung squamous cell carcinoma, *PAAD* Pancreatic adenocarcinoma, *PRAD* Prostate adenocarcinoma, *PCPG* Pheochromocytoma and Paraganglioma, *READ* Rectum ade-nocarcinoma, *SARC* Sarcoma, *SKCM* Skin cutaneous melanoma, *THCA* Thyroid carcinoma, *THYM* Thymoma, *STAD* Stomach adeno-carcinoma, *UCEC* Uterine corpus endometrial carcinoma

reduction of protein expression in tumors ($p < 0.001$) with the only exception of pancreatic adenocarcinoma, glioblastoma multiforme, and hepatocellular carcinoma, where its levels were higher ($p < 0.001$), compared to controls (Fig. 2). The reduced expression of AMBP in seven of ten cancers investigated, with respect to controls, may further support previous findings suggesting its involvement as anti-cancer and anti-metastatic agent. Although interesting, these analyses present some limitations as they are referred to the AMBP gene. Indeed, as UALCAN does not provide any access to mass spectrometry information, discriminating between bikunin and alpha-1-microglobulin (the other

Table 1 AMBP expression levels across cancers

		Protein expression*				mRNA**			
		n	Median	Interquartile range	p value***	n	Median	Interquartile range	p value***
Breast cancer	Normal	18	2.087	1.495 − 2.267	< 0.001	114	0.354	0.11 − 0.695	n.s
	Tumor	125	0.04	− 0.57 − 0.798		1097	0.346	0.102 − 1.23	
Colon cancer	Normal	100	2.757	2.084 − 3.366	< 0.001	41	0.158	0.095 − 0.255	< 0.001
	Tumor	97	− 0.015	− 0.606 − 0.631		286	0.44	0.136 − 1.466	
Ovarian cancer	Normal	25	2.197	1.566 − 2.653	< 0.001	n.d			
	Tumor	100	− 0.039	− 0.564 − 0.554					
Clear cell renal cell carcinoma	Normal	84	1.775	0.762 − 2.371	< 0.001	72	9.095	6.738 − 12.064	n.s
	Tumor	110	0	− 0.639 − 0.682		533	0.896	0.342 − 1.828	
Uterine corpus endometrial carcinoma	Normal	31	2.424	1.95 − 3.263	< 0.001	35	0.195	0.063 − 0.462	< 0.001
	Tumor	100	− 0.045	− 0.53 − 0.43		546	0.804	0.184 − 2.43	
Lung adenocarcinoma	Normal	111	2.452	1.782 − 3.132	< 0.001	59	0.693	0.513 − 1.001	< 0.001
	Tumor	111	− 0.009	− 0.543 − 0.667		515	0.642	0.148 − 2.445	
Pancreatic adenocarcinoma	Normal	74	− 0.722	− 1.273 − − 0.431	< 0.001	4	160.179	80.194 − 170.933	n.s
	Tumor	137	0	− 0.56 − 0.823		178	20.558	3.882 − 177.425	
Head and neck squamous carcinoma	Normal	71	2.866	2.403 − 3.637	< 0.001	n.d			
	Tumor	108	− 0.016	− 0.683 − 0.654					
Glioblastoma multiforme	Normal	10	− 1.801	− 1.934 − − 1.753	< 0.001	5	0.04	0.036 − 0.046	n.s
	Tumor	99	− 0.018	− 0.693 − 0.585		156	0	0 − 0.035	
Hepatocellular carcinoma	Normal	165	− 0.744	− 1.129 − − 0.358	< 0.001	50	7414.869	6459.472 − 8603.095	< 0.001
	Tumor	165	0	− 0.814 − 0.585		371	4913.81	3358.501 − 7136.313	

*Data obtained from Clinical Proteomic Tumor Analysis Consortium (CPTAC) and the International Cancer Proteogenome Consortium (ICPC) datasets, analyzed with the web resource UALCAN (http://ualcan.path.uab.edu/cgi-bin/Pan-cancer-CPTAC.pl?genenam=AMBP)

**Data for cancers included in The Cancer Genome Atlas (TCGA) program, analyzed with the web resource UALCAN (http://ualcan.path.uab.edu/cgi-bin/Pan-cancer.pl?genenam=AMBP)

***Estimated by Student's t-test considering unequal variance

*N.s.* not significant, *n.d.* not determined

protein product of AMBP translation/processing) expression is not possible.

Transcript expression data on 8609 samples across twenty-four types of cancer and 738 controls were obtained (Fig. 3), evidencing highly significant differences between tumors and controls, estimated by Student's t-test, for bladder urothelial carcinoma (BLCA), cervical squamous cell carcinoma (CESC), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), kidney chromophobe (KICH), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), thyroid carcinoma (THCA), stomach adenocarcinoma (STAD), uterine corpus endometrial carcinoma (UCEC) (p < 0.001), and a significant difference for Breast invasive carcinoma (BRCA) (p < 0.05), whereas no differences were found for glioblastoma multiforme (GBM), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), lung squamous cell carcinoma (LUSC), pancreatic adenocarcinoma (PAAD),

sarcoma (SARC). In respect to pheochromocytoma and paraganglioma (PCPG), skin cutaneous melanoma (SKCM), and thymoma (THYM), statistical tests were not applicable because of the low number of controls.

The association between AMBP gene expression and patient's survival in thirty-two different cancer types was also investigated, showing a significant negative impact of high AMBP expression (expression value > 3rd quartile) in brain lower grade glioma (LGG), GBM, KIRP, mesothelioma (MESO), and THCA (Fig. 4). No association was found with regards to the other twenty-seven tumors evaluated (Supplementary Fig. 1).

By comparing protein expression with mRNA levels (whenever possible), widespread discordant patterns were evidenced (Table 1), as also reported in an early work by Sánchez et al. on mouse embryogenesis [21], underling once more that steady-state transcript abundances only partially predict protein levels because of the post-transcriptional, translational, and post-translational regulatory mechanisms occurring after mRNAs are made [75].
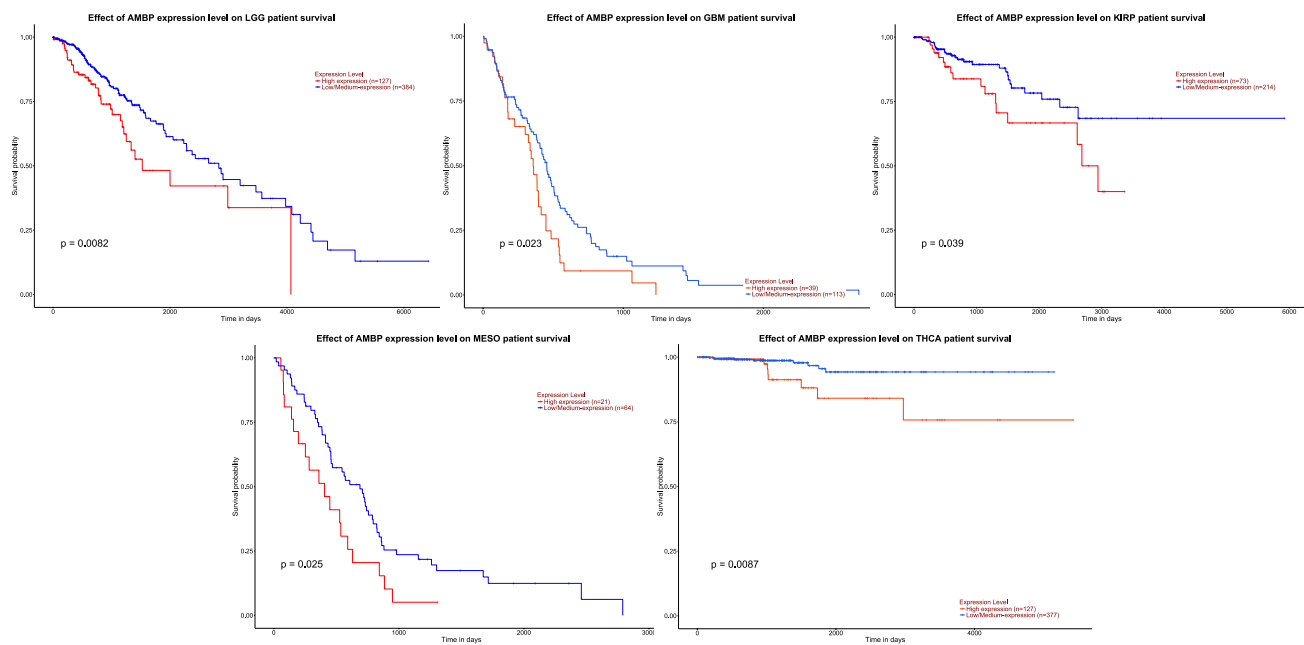
Fig. 4 Kaplan–Meier plots showing a significant association between AMBP gene expression and patient's survival probability in five different cancer types, obtained by the web resource UALCAN. Patients are sorted according to either high (expression value > 3rd quartile) or low AMBP expression levels. Significance is measured by log rank test (p-values < 0.05 are considered to be significant). *LGG* Brain lower grade glioma, *GBM* Glioblastoma multiforme, *KIRP* Kidney renal papillary cell carcinoma, *MESO* Mesothelioma, *THCA* Thyroid carcinoma

Conclusions

Recent technological advances in mass spectrometry-based proteomics have provided researchers with a huge amount of information allowing for large-scale surveys of the cancer proteome. By using the web-tool UALCAN, we analyzed the freely available proteogenomic data for evaluating bikunin expression across several types of tumors showing common patterns, particularly at the protein level. This represents an interesting starting point that should foster further research focusing on the evaluation of the diagnostic and/or prognostic potential of bikunin, for example by analyzing its levels at different tumor stages, both in tissue and in plasma.

Declarations

References

1. Karamanos NK, Theocharis AD, Piperigkou Z, Manou D, Passi A, Skandalis SS, Vynios DH, Orian-Rousseau V, Ricard-Blum S, Schmelzer CEH, Duca L, Durbeej M, Afratis NA, Troeberg L, Franchi M, Masola V, Onisto M (2021) A guide to the composition and functions of the extracellular matrix. FEBS J 288(24):6850–6912. https://doi.org/10.1111/febs.15776

2. Yuzhalin AE, Lim SY, Kutikhin AG (1870) Gordon-Weeks AN (2018) Dynamic matrisome: ECM remodeling factors licensing cancer progression and metastasis. Biochim Biophys Acta 2:207–228. https://doi.org/10.1016/j.bbcan.2018.09.002

3. Karamanos NK, Piperigkou Z, Passi A, Gotte M, Rousselle P, Vlodavsky I (2021) Extracellular matrix-based cancer targeting. Trends Mol Med 27(10):1000–1013. https://doi.org/10.1016/j.molmed.2021.07.009

4. Mahmood N, Mihalcioiu C, Rabbani SA (2018) Multifaceted role of the Urokinase-type Plasminogen Activator (uPA) and its Receptor (uPAR): diagnostic, prognostic, and therapeutic applications. Front Oncol 8:24. https://doi.org/10.3389/fonc.2018.00024

5. Aguirre Ghiso JA, Alonso DF, Farias EF, Gomez DE, de Kier Joffe EB (1999) Deregulation of the signaling pathways controlling urokinase production. Its relationship with the invasive phenotype. Eur J Biochem 263(2):295–304. https://doi.org/10.1046/j.1432-1327.1999.00507.x

6. Kobayashi H, Suzuki M, Hirashima Y, Terao T (2003) The protease inhibitor bikunin, a novel anti-metastatic agent. Biol Chem 384(5):749–754. https://doi.org/10.1515/BC.2003.083

7. Kobayashi H, Suzuki M, Tanaka Y, Kanayama N, Terao T (2003) A Kunitz-type protease inhibitor, bikunin, inhibits ovarian cancer cell invasion by blocking the calcium-dependent transforming growth factor-beta 1 signaling cascade. J Biol Chem 278(10):7790–7799. https://doi.org/10.1074/jbc.M210407200

8. Sun ZJ, Yu T, Chen JS, Sun X, Gao F, Zhao XL, Luo J (2010) Effects of ulinastatin and cyclophosphamide on the growth of xenograft breast cancer and expression of CXC chemokine receptor 4 and matrix metalloproteinase-9 in cancers. J Int Med Res 38(3):967–976. https://doi.org/10.1177/147323001003800323

9. Gao F, Sun Z, Sun X, Zhang Y, Wang H, Zhong B, Luo J, Zhao X (2013) Ulinastatin exerts synergistic effects with taxotere and inhibits invasion and metastasis of breast cancer by blocking angiogenesis and the epithelial-mesenchymal transition. Cancer Biother Radiopharm 28(3):218–225. https://doi.org/10.1089/cbr.2011.1122

10. Imanari T, Shinbo A, Ochiai H, Ikei T, Koshiishi I, Toyoda H (1992) Study on proteoglycans having low-sulfated chondroitin 4-sulfate in human urine and serum. J Pharmacobiodyn 15(5):231–237. https://doi.org/10.1248/bpb1978.15.231

11. Fries E, Blom AM (2000) Bikunin–not just a plasma proteinase inhibitor. Int J Biochem Cell Biol 32(2):125–137. https://doi.org/10.1016/s1357-2725(99)00125-9

12. Zinellu A, Pisanu S, Zinellu E, Lepedda AJ, Cherchi GM, Sotgia S, Carru C, Deiana L, Formato M (2007) A novel LIF-CE method for the separation of hyaluronan- and chondroitin sulfate-derived disaccharides: Application to structural and quantitative analyses of human plasma low- and high-charged chondroitin sulfate isomers. Electrophoresis 28(14):2439–2447. https://doi.org/10.1002/elps.200600668

13. Enghild JJ, Thogersen IB, Cheng F, Fransson LA, Roepstorff P, Rahbek-Nielsen H (1999) Organization of the inter-alpha-inhibitor heavy chains on the chondroitin sulfate originating from Ser(10) of bikunin: posttranslational modification of IalphaI-derived bikunin. Biochemistry 38(36):11804–11813. https://doi.org/10.1021/bi9908540

14. Kaczmarczyk A, Blom AM, Alston-Smith J, Sjoquist M, Fries E (2005) Plasma bikunin: half-life and tissue uptake. Mol Cell Biochem 271(1–2):61–67. https://doi.org/10.1007/s11010-005-5282-3

15. Chi L, Wolff JJ, Laremore TN, Restaino OF, Xie J, Schiraldi C, Toida T, Amster IJ, Linhardt RJ (2008) Structural analysis of bikunin glycosaminoglycan. J Am Chem Soc 130(8):2617–2625. https://doi.org/10.1021/ja0778500

16. Vetr H, Gebhard W (1990) Structure of the human alpha 1-microglobulin-bikunin gene. Bio Chem Hoppe-Seyler 371(12):1185–1196. https://doi.org/10.1515/bchm3.1990.371.2.1185

17. Kaumeyer JF, Polazzi JO, Kotick MP (1986) The mRNA for a proteinase inhibitor related to the HI-30 domain of inter-alpha-trypsin inhibitor also encodes alpha-1-microglobulin (protein HC). Nucleic Acids Res 14(20):7839–7850. https://doi.org/10.1093/nar/14.20.7839

18. Morelle W, Capon C, Balduyck M, Sautiere P, Kouach M, Michalski C, Fournet B, Mizon J (1994) Chondroitin sulphate covalently cross-links the three polypeptide chains of inter-alpha-trypsin inhibitor. Eur J Biochem 221(2):881–888. https://doi.org/10.1111/j.1432-1033.1994.tb18803.x

19. Zhuo L, Hascall VC, Kimata K (2004) Inter-alpha-trypsin inhibitor, a covalent protein-glycosaminoglycan-protein complex. J Biol Chem 279(37):38079–38082. https://doi.org/10.1074/jbc.R300039200

20. Zhuo L, Kimata K (2008) Structure and function of inter-alpha-trypsin inhibitor heavy chains. Connect Tissue Res 49(5):311–320. https://doi.org/10.1080/03008200802325458

21. Sanchez D, Martinez S, Lindqvist A, Akerstrom B, Falkenberg C (2002) Expression of the AMBP gene transcript and its two protein products, alpha(1)-microglobulin and bikunin, in mouse embryogenesis. Mech Dev 117(1–2):293–298. https://doi.org/10.1016/s0925-4773(02)00202-2

22. Zhuo L, Yoneda M, Zhao M, Yingsung W, Yoshida N, Kitagawa Y, Kawamura K, Suzuki T, Kimata K (2001) Defect in SHAP-hyaluronan complex causes severe female infertility. A study by inactivation of the bikunin gene in mice. J Biol Chem 276(11):7693–7696. https://doi.org/10.1074/jbc.C000899200

23. Sanggaard KW, Sonne-Schmidt CS, Krogager TP, Lorentzen KA, Wisniewski HG, Thogersen IB, Enghild JJ (2008) The transfer of heavy chains from bikunin proteins to hyaluronan requires both TSG-6 and HC2. J Biol Chem 283(27):18530–18537. https://doi.org/10.1074/jbc.M800874200

24. Colon E, Shytuhina A, Cowman MK, Band PA, Sanggaard KW, Enghild JJ, Wisniewski HG (2009) Transfer of inter-alpha-inhibitor heavy chains to hyaluronan by surface-linked hyaluronan-TSG-6 complexes. J Biol Chem 284(4):2320–2331. https://doi.org/10.1074/jbc.M807183200

25. Lamkin E, Cheng G, Calabro A, Hascall VC, Joo EJ, Li L, Linhardt RJ, Lauer ME (2015) Heavy chain transfer by tumor necrosis factor-stimulated gene 6 to the bikunin proteoglycan. J Biol Chem 290(8):5156–5166. https://doi.org/10.1074/jbc.M114.636258

26. Lord MS, Day AJ, Youssef P, Zhuo L, Watanabe H, Caterson B, Whitelock JM (2013) Sulfation of the bikunin chondroitin sulfate chain determines heavy chain hyaluronan complex formation. J Biol Chem 288(32):22930–22941. https://doi.org/10.1074/jbc.M112.404186

27. Kobayashi H, Gotoh J, Hirashima Y, Fujie M, Sugino D, Terao T (1995) Inhibitory effect of a conjugate between human urokinase and urinary trypsin inhibitor on tumor cell invasion in vitro. J Biol Chem 270(14):8361–8366. https://doi.org/10.1074/jbc.270.14.8361

28. Kobayashi H, Shinohara H, Gotoh J, Fujie M, Fujishiro S, Terao T (1995) Anti-metastatic therapy by urinary trypsin inhibitor in

combination with an anti-cancer agent. Br J Cancer 72(5):1131–1137. https://doi.org/10.1038/bjc.1995.476

29. Matsuzaki H, Kobayashi H, Yagyu T, Wakahara K, Kondo T, Kurita N, Sekino H, Inagaki K, Suzuki M, Kanayama N, Terao T (2004) Bikunin inhibits lipopolysaccharide-induced tumor necrosis factor alpha induction in macrophages. Clin Diagn Lab Immunol 11(6):1140–1147. https://doi.org/10.1128/CDLI.11.6.1140-1147.2004

30. Wakahara K, Kobayashi H, Yagyu T, Matsuzaki H, Kondo T, Kurita N, Sekino H, Inagaki K, Suzuki M, Kanayama N, Terao T (2005) Bikunin suppresses lipopolysaccharide-induced lethality through down-regulation of tumor necrosis factor- alpha and interleukin-1 beta in macrophages. J Infect Dis 191(6):930–938. https://doi.org/10.1086/428134

31. Maehara K, Kanayama N, Halim A, el Maradny E, Oda T, Fujita M, Terao T (1995) Down-regulation of interleukin-8 gene expression in HL60 cell line by human Kunitz-type trypsin inhibitor. Biochem Biophys Res Commun 206(3):927–934. https://doi.org/10.1006/bbrc.1995.1131

32. Kanayama N, el Maradny E, Halim A, Liping S, Maehara K, Kajiwara Y, Terao T (1995) Urinary trypsin inhibitor prevents uterine muscle contraction by inhibition of Ca++ influx. Am J Obstet Gynecol 173(1):192–199. https://doi.org/10.1016/0002-9378(95)90189-2

33. Kanayama N, Maehara K, She L, Belayet HM, Khatun S, Tokunaga N, Terao T (1998) Urinary trypsin inhibitor suppresses vascular smooth muscle contraction by inhibition of Ca2+ influx. Biochem Biophys Acta 1381(2):139–146. https://doi.org/10.1016/s0304-4165(98)00022-1

34. Hiyama A, Takeda J, Kotake Y, Morisaki H, Fukushima K (1997) A human urinary protease inhibitor (ulinastatin) inhibits neutrophil extracellular release of elastase during cardiopulmonary bypass. J Cardiothorac Vasc Anesth 11(5):580–584. https://doi.org/10.1016/s1053-0770(97)90008-2

35. Kobayashi H, Shibata K, Fujie M, Terao T (1998) Urinary trypsin inhibitor reduces the release of histamine from rat peritoneal mast cells. J Lab Clin Med 131(4):375–385. https://doi.org/10.1016/s0022-2143(98)90189-5

36. Kato K (1995) Effect of human urinary trypsin inhibitor (ulinastatin) on inflammatory mediators from leukocytes : possible role in the prevention of SIRS. Igaku To Yakugaku 34:499–506

37. Cowan B, Baron O, Crack J, Coulber C, Wilson GJ, Rabinovitch M (1996) Elafin, a serine elastase inhibitor, attenuates postcardiac transplant coronary arteriopathy and reduces myocardial necrosis in rabbits afer heterotopic cardiac transplantation. J Clin Investig 97(11):2452–2468. https://doi.org/10.1172/JCI118692

38. Atmani F, Glenton PA, Khan SR (1999) Role of inter-alpha-inhibitor and its related proteins in experimentally induced calcium oxalate urolithiasis. Localization of proteins and expression of bikunin gene in the rat kidney. Urological Res 27(1):63–67. https://doi.org/10.1007/s002400050090

39. Khan SR, Kok DJ (2004) Modulators of urinary stone formation. Front Biosci 9:1450–1482. https://doi.org/10.2741/1347

40. Nakakuki M, Yamasaki F, Shinkawa T, Kudo M, Watanabe M, Mizota M (1996) Protective effect of human ulinastatin against gentamicin-induced acute renal failure in rats. Can J Physiol Pharmacol 74(1):104–111

41. Kato Y, Kudo M, Shinkawa T, Mochizuki H, Isaji M, Shiromizu I, Hoshida K (1998) Role of O-linked carbohydrate of human urinary trypsin inhibitor on its lysosomal membrane-stabilizing property. Biochem Biophys Res Commun 243(2):377–383. https://doi.org/10.1006/bbrc.1998.8100

42. Pugia MJ, Lott JA (2005) Pathophysiology and diagnostic value of urinary trypsin inhibitors. Clin Chem Lab Med 43(1):1–16. https://doi.org/10.1515/CCLM.2005.001

43. Wei X, Zhu X, Jiang L, Long M, Du Y (2021) Recent research progress on the role of ulinastatin in chronic kidney disease. Nephrology 26(9):708–714. https://doi.org/10.1111/nep.13906

44. Lepedda AJ, De Muro P, Capobianco G, Formato M (2020) Role of the small proteoglycan bikunin in human reproduction. Hormones 19(2):123–133. https://doi.org/10.1007/s42000-019-00149-x

45. Jortani SA, Pugia MJ, Elin RJ, Thomas M, Womack EP, Cast T, Valdes R Jr (2004) Sensitive noninvasive marker for the diagnosis of probable bacterial or viral infection. J Clin Lab Anal 18(6):289–295. https://doi.org/10.1002/jcla.20040

46. Lin SD, Endo R, Kuroda H, Kondo K, Miura Y, Takikawa Y, Kato A, Suzuki K (2004) Plasma and urine levels of urinary trypsin inhibitor in patients with chronic liver diseases and hepatocellular carcinoma. J Gastroenterol Hepatol 19(3):327–332. https://doi.org/10.1111/j.1440-1746.2003.03221.x

47. Matsuzaki H, Kobayashi H, Yagyu T, Wakahara K, Kondo T, Kurita N, Sekino H, Inagaki K, Suzuki M, Kanayama N, Terao T (2005) Plasma bikunin as a favorable prognostic factor in ovarian cancer. J Clin Oncol 23(7):1463–1472. https://doi.org/10.1200/JCO.2005.03.010

48. Tsui KH, Tang P, Lin CY, Chang PL, Chang CH, Yung BY (2010) Bikunin loss in urine as useful marker for bladder carcinoma. J Urol 183(1):339–344. https://doi.org/10.1016/j.juro.2009.08.109

49. Mizon C, Piva F, Queyrel V, Balduyck M, Hachulla E, Mizon J (2002) Urinary bikunin determination provides insight into proteinase/proteinase inhibitor imbalance in patients with inflammatory diseases. Clin Chem Lab Med 40(6):579–586. https://doi.org/10.1515/CCLM.2002.100

50. Lepedda AJ, De Muro P, Capobianco G, Formato M (2017) Significance of urinary glycosaminoglycans/proteoglycans in the evaluation of type 1 and type 2 diabetes complications. J Diabetes Complications 31(1):149–155. https://doi.org/10.1016/j.jdiacomp.2016.10.013

51. Lepedda AJ, Fancellu L, Zinellu E, De Muro P, Nieddu G, Deiana GA, Canu P, Concolino D, Sestito S, Formato M, Sechi G (2013) Urine bikunin as a marker of renal impairment in Fabry's disease. Biomed Res Int 2013:205948. https://doi.org/10.1155/2013/205948

52. Lepedda AJ, Nieddu G, Rocchiccioli S, Fresu P, De Muro P, Formato M (2013) Development of a method for urine bikunin/urinary trypsin inhibitor (UTI) quantitation and structural characterization: Application to type 1 and type 2 diabetes. Electrophoresis 34(22–23):3227–3233. https://doi.org/10.1002/elps.201300384

53. Lepedda AJ, Nieddu G, Rocchiccioli S, Ucciferri N, Idini M, De Muro P, Formato M (2018) Levels of urinary trypsin inhibitor and structure of its chondroitin sulphate moiety in type 1 and type 2 diabetes. J Diabetes Res 2018:9378515. https://doi.org/10.1155/2018/9378515

54. Capon C, Mizon C, Lemoine J, Rodie-Talbere P, Mizon J (2003) In acute inflammation, the chondroitin-4 sulphate carried by bikunin is not only longer, it is also undersulphated. Biochimie 85(1–2):101–107. https://doi.org/10.1016/s0300-9084(03)00066-x

55. Hettinger AM, Allen MR, Zhang BR, Goad DW, Malayer JR, Geisert RD (2001) Presence of the acute phase protein, bikunin, in the endometrium of gilts during estrous cycle and early pregnancy. Biol Reprod 65(2):507–513. https://doi.org/10.1095/biolreprod65.2.507

56. Mizon C, Mairie C, Balduyck M, Hachulla E, Mizon J (2001) The chondroitin sulfate chain of bikunin-containing proteins in the inter-alpha-inhibitor family increases in size in inflammatory diseases. Eur J Biochem 268(9):2717–2724. https://doi.org/10.1046/j.1432-1327.2001.02168.x

57. Kobayashi H, Suzuki M, Tanaka Y, Hirashima Y, Terao T (2001) Suppression of urokinase expression and invasiveness by urinary

trypsin inhibitor is mediated through inhibition of protein kinase C- and MEK/ERK/c-Jun-dependent signaling pathways. J Biol Chem 276(3):2015–2022. https://doi.org/10.1074/jbc.M007650200

58. Hirashima Y, Kobayashi H, Suzuki M, Tanaka Y, Kanayama N, Fujie M, Nishida T, Takigawa M, Terao T (2001) Characterization of binding properties of urinary trypsin inhibitor to cell-associated binding sites on human chondrosarcoma cell line HCS-2/8. J Biol Chem 276(17):13650–13656. https://doi.org/10.1074/jbc.M009906200

59. Kobayashi H, Hirashima Y, Sun GW, Fujie M, Shibata K, Tamotsu S, Miura K, Sugino D, Tanaka Y, Kondo S, Terao T (1998) Identification and characterization of the cell-associated binding protein for urinary trypsin inhibitor. Biochem Biophys Acta 1383(2):253–268. https://doi.org/10.1016/s0167-4838(97)00215-x

60. Suzuki M, Kobayashi H, Tanaka Y, Hirashima Y, Terao T (2001) Structure and function analysis of urinary trypsin inhibitor (UTI): identification of binding domains and signaling property of UTI by analysis of truncated proteins. Biochem Biophys Acta 1547(1):26–36. https://doi.org/10.1016/s0167-4838(01)00167-4

61. Suzuki M, Kobayashi H, Fujie M, Nishida T, Takigawa M, Kanayama N, Terao T (2002) Kunitz-type protease inhibitor bikunin disrupts phorbol ester-induced oligomerization of CD44 variant isoforms containing epitope v9 and subsequently suppresses expression of urokinase-type plasminogen activator in human chondrosarcoma cells. J Biol Chem 277(10):8022–8032. https://doi.org/10.1074/jbc.M108545200

62. Yagyu T, Kobayashi H, Wakahara K, Matsuzaki H, Kondo T, Kurita N, Sekino H, Inagaki K, Suzuki M, Kanayama N, Terao T (2004) A kunitz-type protease inhibitor bikunin disrupts ligand-induced oligomerization of receptors for transforming growth factor (TGF)-beta and subsequently suppresses TGF-beta signalings. FEBS Lett 576(3):408–416. https://doi.org/10.1016/j.febslet.2004.09.048

63. Suzuki M, Kobayashi H, Tanaka Y, Hirashima Y, Kanayama N, Takei Y, Saga Y, Suzuki M, Itoh H, Terao T (2003) Suppression of invasion and peritoneal carcinomatosis of ovarian cancer cell line by overexpression of bikunin. Int J Cancer 104(3):289–302. https://doi.org/10.1002/ijc.10950

64. Wang J, Chen X, Su L, Zhu Z, Wu W, Zhou Y (2016) Suppressive effects on cell proliferation and motility in gastric cancer SGC-7901 cells by introducing ulinastatin in vitro. Anticancer Drugs 27(7):651–659. https://doi.org/10.1097/CAD.0000000000000378

65. Tanaka Y, Kobayashi H, Suzuki M, Kanayama N, Suzuki M, Terao T (2004) Upregulation of bikunin in tumor-infiltrating macrophages as a factor of favorable prognosis in ovarian cancer. Gynecol Oncol 94(3):725–734. https://doi.org/10.1016/j.ygyno.2004.06.012

66. Yagyu T, Kobayashi H, Matsuzaki H, Wakahara K, Kondo T, Kurita N, Sekino H, Inagaki K (2006) Enhanced spontaneous metastasis in bikunin-deficient mice. Int J Cancer 118(9):2322–2328. https://doi.org/10.1002/ijc.21293

67. Kobayashi H, Yagyu T, Inagaki K, Kondo T, Suzuki M, Kanayama N, Terao T (2004) Therapeutic efficacy of once-daily oral administration of a Kunitz-type protease inhibitor, bikunin, in a mouse model and in human cancer. Cancer 100(4):869–877. https://doi.org/10.1002/cncr.20034

68. McIntosh M (2005) Could high plasma bikunin predict a favorable outcome in ovarian cancer? Nat Clin Pract Oncol 2(10):502–503. https://doi.org/10.1038/ncponc0315

69. Sekikawa S, Onda T, Miura N, Nomura T, Takano N, Shibahara T, Honda K (2018) Underexpression of alpha-1-microglobulin/bikunin precursor predicts a poor prognosis in oral squamous cell carcinoma. Int J Oncol 53(6):2605–2614. https://doi.org/10.3892/ijo.2018.4581

70. Chandrashekar DS, Bashel B, Balasubramanya SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi B, Varambally S (2017) UALCAN: a portal for facilitating tumor subgroup gene expression and survival analyses. Neoplasia 19(8):649–658. https://doi.org/10.1016/j.neo.2017.05.002

71. Chandrashekar DS, Karthikeyan SK, Korla PK, Patel H, Shovon AR, Athar M, Netto GJ, Qin ZS, Kumar S, Manne U, Creighton CJ, Varambally S (2022) UALCAN: An update to the integrated cancer data analysis platform. Neoplasia 25:18–27. https://doi.org/10.1016/j.neo.2022.01.001

72. Chen F, Chandrashekar DS, Varambally S, Creighton CJ (2019) Pan-cancer molecular subtypes revealed by mass-spectrometry-based proteomic characterization of more than 500 human cancers. Nat Commun 10(1):5679. https://doi.org/10.1038/s41467-019-13528-0

73. Zhang Y, Chen F, Chandrashekar DS, Varambally S, Creighton CJ (2022) Proteogenomic characterization of 2002 human cancers reveals pan-cancer molecular subtypes and associated pathways. Nat Commun 13(1):2669. https://doi.org/10.1038/s41467-022-30342-3

74. Hutter C, Zenklusen JC (2018) The cancer genome atlas: creating lasting value beyond its data. Cell 173(2):283–285. https://doi.org/10.1016/j.cell.2018.03.042

75. Vogel C, Marcotte EM (2012) Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat Rev Genet 13(4):227–232. https://doi.org/10.1038/nrg3185