Exp Oncol 2014
36, 2, 107–111

Experimental ONCOLOGY

REDUCED GENE EXPRESSION OF *BIKUNIN* AS A PROGNOSTIC MARKER FOR RENAL CELL CARCINOMA

E. Bayraktar[1], M. Igci[1,], S. Erturhan[2], Y.Z. Igci[1], M. Karakok[3], B. Gogebakan[4], M. Ulasli[1], E.A. Cakmak[1], A. Arslan[1]*

[1]*Department of Medical Biology, Faculty of Medicine, University of Gaziantep, Gaziantep 27310, Turkey*
[2]*Department of Urology, Faculty of Medicine, University of Gaziantep, Gaziantep 27310, Turkey*
[3]*Department of Pathology, Faculty of Medicine, University of Gaziantep, Gaziantep 27310, Turkey*
[4]*Department of Medical Biology, Faculty of Medicine, Mustafa Kemal University, Hatay 31030, Turkey*

***Aim*: Experimental and clinical studies showed that bikunin, a Kunitz-type protease inhibitor, found in urine and amniotic fluid has a role in spread of tumor cells by providing a significant reduction in the levels of urokinase-type plasminogen activator (uPA) and its specific receptor urokinase-type plasminogen activator receptor (uPAR). The aim of this study was to investigate expression of bikunin at the mRNA level and screen for mutations in exon sequence in renal cell carcinoma (RCC) tissues. *Materials and Methods*: Total RNA and DNA were extracted from paired normal and tumor tissues of total 50 RCC (11 papillary, 8 chromophobe, 26 clear cell, and 5 other types) patients (23 females, mean age: 53.55 ± 14.17; 27 males mean age: 62.1 ± 7.92). Bikunin mRNA levels were detected using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Mutational screening was performed by using single strand conformation polymorphism (SSCP) method and nucleotide sequence analysis. *Results*: There was a statistically significant decrease in the 25 (50%) of tumor tissues comparing to normal tissues in terms of mRNA levels of bikunin (Wilcoxon signed rank test, $p = 0.0337$). According to the classification based on subtypes of RCC; clear cell RCC samples displayed a reduced gene expression ($p = 0.0148$). Additionally, the patients with the age above 50 had low bikunin expression. The SNP rs80057939 spanning 4th exon of bikunin was detected in 13 tumor tissues. However, it was not statistically significant ($p > 0.05$). *Conclusion*: Decreased bikunin mRNA level in renal cells might be associated with poor prognosis of renal carcinoma. Therefore, gene constructs or exogenous administration of bikunin might be a potential adjuvant therapy for RCC treatment.**

***Key Words*: Bikunin, nucleotide sequence analysis, prognostic marker, renal cell carcinoma, semi-quantitative RT-PCR.**

Kidney neoplasms account for approximately 2% of all cancers [1], and clear cell renal cell carcinoma (RCC), papillary RCC, oncocytoma, and chromophobe RCC are the most frequent types of renal cancers [2]. Several genes have been associated with renal tumor generation, progression, proliferation, invasion, and metastasis by using mutational screening, gene and protein expression analyses [1].

Bikunin, a Kunitz-type protease inhibitor, is found in human amniotic fluid, urine, serum [3], and also known as urinary trypsin inhibitor (uTi), miraclid, mingin, serpin, human inhibitor 30 (HI-30), urinastatin, ulinastatin [4]. Bikunin is mainly expressed in liver tissue, but also expressed at lower concentrations in pancreas, kidney, lung, intestine, stomach, skin, gallbladder, cerebrum, cerebellum, testis, brain, mast cells, and colon suggesting different physiological roles of bikunin [5–11].

Protease inhibitors that contain Kunitz domain function to obstruct protease activities throughout homeostasis, tissue injury, inflammation, and cancer formation and progression [12]. The balance between proteases and protease inhibitors is critical in invasion and metastasis of tumor cells [13]. The expression level of bikunin alters in case of cancer, infection, tissue injury, kidney disease, vascular disease, and diabetes [4]. The concentration of bikunin protein in plasma and urine changes in the same conditions, as well [4].

Bikunin suppresses invasion and metastasis capability of tumor in many cell lines by inhibiting cell-associated plasmin activity as well as by inhibiting uPA-uPAR gene and protein expression levels by suppression of TGF-β1-dependent ERK1/2 activation [14–16]. Bikunin has a proven anti-invasive and anti-metastatic effect in several types of malignant cells [17, 18].

In this study, we aimed to determine the possible association between bikunin and RCC pathogenesis by monitoring the expression level and sequence analysis of bikunin in order to investigate the possible role of bikunin in RCC.

MATERIALS AND METHODS

Normal and tumor tissue specimens. Fifty paired tumor and normal samples of RCC (11 papillary, 8 chromophobe, 26 clear cell, and 5 other subtypes) patients (23 females, mean age: 53.55 ± 14.17; 27 males, mean age: 62.1 ± 7.92) were obtained by primary surgery

Submitted: February 27, 2014.
*Correspondence: Fax: +90 342 3601617;
E-mail: mehriigci@gmail.com, mehriigci@gantep.edu.tr
Abbreviations used: Akt – protein kinase B-alpha; CD10 – membrane metalloendopeptidase; cDNA – complementary DNA; ERK – extracellular signal-regulated kinase; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; HI-30 – human inhibitor 30; MMP – matrix metalloproteinase; PAX2 – paired box gene 2; PAX8 – paired box gene 8; PI3K – phosphoinositide 3-kinases; RCC – renal cell carcinoma; RT-PCR – reverse transcriptase PCR; SNP – single nucleotide polymorphism; SSCP – single strand conformation polymorphism; TGF-β1 – transforming growth factor beta-1; uPA – urokinase-type plasminogen activator receptor; uPAR – urokinase-type plasminogen activator receptor; uTi – urinary tripsin inhibitor.

at the Department of Urology, Faculty of Medicine, University of Gaziantep. Informed consent was taken from all participants. The obtained tissues were placed into liquid nitrogen immediately after the surgery and stored at −80 °C until the DNA and RNA were extracted. This study was approved by the local ethical committee, in accordance with the declaration of Helsinki.

DNA & RNA extraction. DNA samples and RNA extraction was carried out upon confirmation by histopathological analysis. DNA samples were obtained by using extraction kits (for DNA: Qiagen, QIAmp DNA Mini Kit, Catalog No. 51306; for RNA: Qiagen RNA Isolation Kit, Catalog No. 74104, Hilden, Germany) according to the instructions of the manufacturer. Quantification of DNA and RNA concentrations were performed by using spectrophotometer (NanoDrop, ND-1000, USA).

Semi-quantitative reverse transcription PCR analysis. cDNAs were synthesized from RNA samples by using AB High Capacity RNA-to-cDNA kit (Catalog No. 4387951, USA) according to manufacturer's instructions. cDNAs were amplified by the expression primers seen in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for the normalization of bikunin gene expression data. The numbers of cycles for bikunin and GAPDH amplifications were 30 and 25, respectively. Expression alterations were demonstrated by agarose gel electrophoresis (2%) in the presence of 0.5 g/mL of ethidium bromide. The image of the agarose gel was captured and quantitated by ImageJ software (v1.46r).

Table 1. The primers used for PCR amplification

Primers	Primer sequences	Expected size of PCR products (bp)	Annealing temperatures (°C)
BKN1-F (AMBP-7)	CAGAAATCTGTCCTCTGATGC	307	54.7
BKN1-R (AMBP-7)	AACACCTGCCTTTTCACCC		
BKN2-F (AMBP-8)	ACACTTGGTCAGTGCTGCTG	277	58.7
BKN2-R (AMBP-8)	ATTCGCAGGTGGTTTACTGG		
BKN3-F (AMBP-9)	ATGCCTCTCTCCACTCCACA	255	53.6
BKN3-R (AMBP-9)	TCTCCACCTCGACTCCAAC		
BKN4-F (AMBP-10)	TGTGTCCACAGGTGATGAGG	209	61.3
BKN4-R (AMBP-10)	TCCGCCACCCTGTTAGTTAC		
BKNexFw	CTGCAATCTCCCCATAGTC	230	58.7
BKNexRv	ATCCTCTGACTTGCAGACC		
GAPDHFw	GGTCCACCACCCTGTTGCTGT	456	59.4
GAPDHRv	AGACCACAGTCGATGCCATCAC		

PCR analysis. Primers were designed by using Primer-BLAST (see Table 1). PCR amplifications were performed in 20 µl master mixture containing 2 µl genomic DNA, 20 pmole each of primers, 10× buffer, 2 mM each of nucleotides (dATP, dCTP, dGTP, dTTP) and 0.5 unit of Taq DNA polymerase. PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 45 s, annealing at the temperatures seen in Table 1 for 45 s, and elongation at 72 °C for 45 s. A final extension step was applied at 72 °C for 5 min. PCR experiments were carried out by AB Thermal Cycler. PCR products were electrophoresed in 2.0% agarose gel.

Single strand conformation polymorphism (SSCP) analysis & sequencing. 7–10 µl loading dye (95% formamide, 20 mM EDTA, 0.05% xylene cyanol) was added into PCR tubes containing 3 µl PCR products. Samples were denatured at 95 °C for 5 min and were put on ice to prevent renaturation, and electrophoresed on 7% polyacrylamide gel (49:1 Acrylamide : Bisacrylamide). To visualize DNA bands, silver nitrate staining was used.

In order to confirm SSCP results, the fragments which showed different mobility were excised from the gel and analyzed by ABI 3130 nucleotide sequencer. The obtained sequence was compared with the reference assembly in the NCBI database (Accession number: NT_008470.19).

Statistical analysis. Prior to the statistical evaluation of expression results, D'Agostino & Pearson omnibus normality test, Shapiro — Wilk normality test, and Kolmogorov — Smirnov test were used to examine the normality of the variables' distributions. Subsequently, the expression data was assessed by Wilcoxon signed rank test. The sequencing results were evaluated by *t*-test.

The postoperative period was measured from the surgery to the death or last follow-up. In order to estimate the cumulative survival probabilities among RCC patients, the Kaplan — Meier curve was used. The risk ratio of contributions of covariates such as expression alterations, age, gender, tumor size, and metastatic state were assessed by a Cox proportional hazards model. A 2-tailed value $p < 0.05$ was considered statistically significant in all of the statistical tests. The statistical tests were made by using Graphpad Prism (v.6.02) and SPSS (v.16) softwares.

RESULTS

Gene expression analysis. The median value of the bikunin mRNA to GAPDH mRNA ratio in normal and tumor samples was calculated as 1.072 and the ratio higher than 1.072 was defined as overexpression. Twenty-five of total 50 RCC specimens had lower bikunin gene expression comparing to normals. The distributions of variables' were significant (D'Agostino & Pearson omnibus normality test, Shapiro — Wilk normality test and Kolmogorov — Smirnov test; $p < 0.05$). The bikunin mRNA levels measured in tumor tissue were significantly lower than normal tissue of patients with RCC (Fig. 1, Table 2) ($p = 0.0337$). When the specimens were subdivided according to the subtypes of RCC, the clear cell type of RCC displayed the significantly low expression of bikunin ($p = 0.0021$). Furthermore, in terms of clinicopathologic parameters, only age is observed as a significant covariate in bikunin expression. The mRNA expression level of bikunin of tumor samples that belong to patients above 50 years old was significantly lower than normal samples ($p = 0.0147$). However, metastasis-negative tumors were significantly low expressors of bikunin ($p = 0.0388$). No significant difference in clinical stage, pathological stage, or tumor size was detected for patient group ($p > 0.05$).

There was no significant difference in the survival rates of patients with the low and high expression of bikunin ($p > 0.05$) (Fig. 2). Cox proportional hazards model showed that low level of bikunin expression

(p = 0.028) and age above 50 (p = 0.046) were significant risk factors affecting overall survival (Table 3).

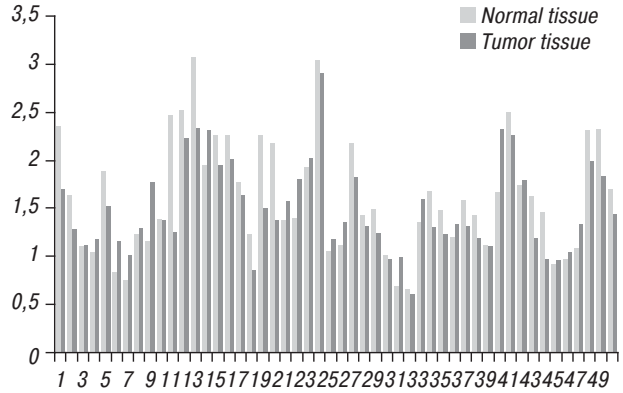

Fig. 1. Comparison of bikunin expression levels in tumor and normal samples. Twenty-five of total were low expressers of bikunin

Table 2. The relationship between clinical parameters and bikunin expression level

Characteristics	Total number	Bikunin mRNA expression		p value
		High	Low	
Age (years)				
< 50	10	5	5	NS
> 50	40	25	15	0.0147
Subtype of RCC				
Classic RCC	10	3	7	0.0488
Papillary RCC	11	8	3	NS
Chromophobe RCC	8	5	3	NS
Clear cell RCC	16	7	9	0.0021
Other	5	2	3	NS
Total RCC	50	25	25	0.0337
Clinical Stage				
T1	1	0	1	NS
T1a	5	4	1	NS
T1b	17	9	8	NS
T2	1	1	0	NS
T2a	14	7	7	NS
T2b	8	5	3	NS
T3	3	3	0	NS
Unknown	1	1	0	NS
Pathological Stage				
T1	13	10	3	NS
T1a	2	1	1	NS
T2	18	11	7	NS
T3	2	1	1	NS
T3a	3	1	2	NS
Unknown	12	6	6	NS
Tumor size (cm)				
< 5	13	6	7	NS
5–10	28	16	12	NS
> 10	9	9	0	NS
Metastasis				
Positive	14	7	7	NS
Negative	36	18	18	0.0388

Note: NS – not significant.

Table 3. Analysis the effects of risk factors on overall survival by Cox proportional hazard model

	P	Hazard	95,0% CI for Hazard		
			Lower	Upper	Wald
Expression Level	0.028	0.107	0.015	0.782	4.853
Age	0.046	1.039	1.001	1.079	3.966
Gender	0.728	1.181	0.462	3.019	0.121
Metastasis	0.316	1.590	0.643	3.932	1.007
Tumor size	0.332	1.060	0.942	1.193	0.332

Note: 95% CI – 95% confidence interval.

SSCP and nucleotide sequence analysis. As a result of SSCP and sequence analyses, 13 of 50 kidney carcinoma samples (26%) displayed A > G substitution which was formerly identified as rs80057939 (p = 0.069) (Fig. 3). rs80057939 is located on the 3'-UTR of bikunin gene and was the only single nucleotide polymorphism (SNP) detected in our samples.

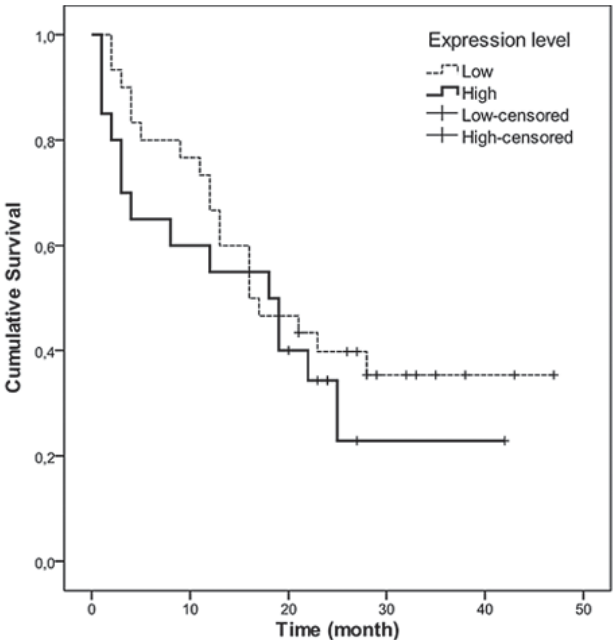

Fig. 2. Impact of expression level of bikunin on cumulative survival was assessed via Kaplan — Meier method. No significant relationship was observed (p > 0.05)

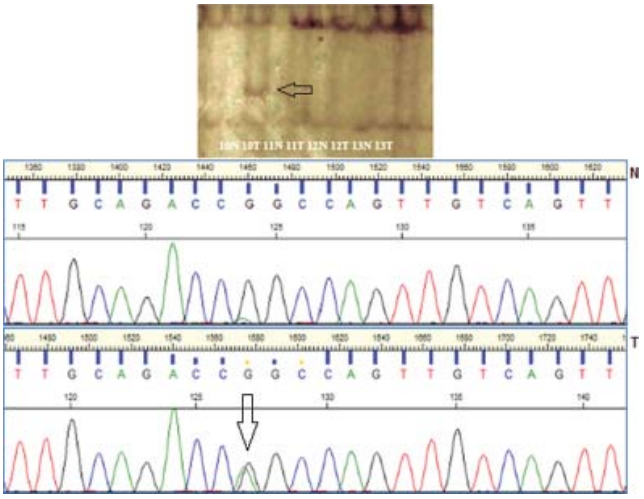

Fig. 3. Gel image of SSCP of exon 4. The band indicated by the arrow was excised from the gel and subjected to sequence analysis. G>A substitution (rs80057939) was observed in 13 of 50 (26%) tumor samples (p > 0.05)

DISCUSSION

RCC, with different histopathological features, genetic expression and clinical behavior, is a heterogeneous disease with many subtypes [2, 19]. The most common subtypes of RCC are clear cell, papillary, and chromophobe comprising approximately 70 to 80%, 14 to 17%, and 4 to 8% of all, respectively [20]. Several numbers of immunomarkers have been utilized for early detection and prognosis of renal tumors. However, most of them are not specific despite being sensitive. PAX2, PAX8, CD10, and RCC marker are the most preferred markers for diagnosis of RCC [20].

In recent years, experimental and clinical results showed that bikunin takes part in many biological processes [21–23]. Many proteolytic enzymes such as uPA [24–26], cathepsin [27] and various matrix metalloproteins (MMPs) [28–31] play crucial role in tumor metastasis. During proliferation of tumor cells, control mechanism of proteases are lost [32]. Bikunin exhibits

anti-metastatic functions in animals and humans. Several bikunin-regulated genes were identified and of these PI3K is considered to be a critical bikunin target gene [33]. Kobayashi *et al*. demonstrated that in HRA cells after TGF-β1 treatment Akt is phosphorylated and that reduced PI3K expression due to inhibition of PI3K activity prevent the uPA up-regulation and invasion in HRA cell line [34].

In a study analyzing 13 different tumor tissues, in terms of expression levels of the genes of inter-α-inhibitor family, bikunin was reported to be expressed abundantly in all of the tumor tissues except the kidney tissue which displayed bikunin down-regulation in a ratio of 90% [35]. This interesting data can also give rise to an important point that is mostly unclear: the reason for abundant expression of bikunin especially specific to kidney tissue. One of the possible explanations may be the tendency for stone formation in the urine that triggers expression of bikunin as a calcium chelator to struggle with calcium oxalate (CaOx) kidney stones in a continual manner [36]. However, the functions and biological significance of bikunin remains to be established.

The experiments measured the protein level of bikunin in urine of kidney cancer patients revealed that the amount of bikunin in urine varies in consistent with the number of cancer cells [37]. However, there is no such a detailed study at the molecular level performed with RCC patients.

To demonstrate the effect of bikunin expression at the mRNA level in RCC, we performed semi-quantitative RT-PCR and sequencing experiments. The relationship between low *bikunin* expression and poor prognosis of RCC was independent of gender, tumor size, pathological stage or clinical stage of patients ($p > 0.05$) (see Table 2). Interestingly, *bikunin* down-regulation was common among metastasis-negative patients ($p = 0.0388$) (see Table 2). The small number of our patient group may give rise to this result. Further studies with larger groups are required to clarify the effect of metastatic state in RCC.

In a Cox proportional hazards model of the risk factors, low bikunin expression was an independent factor for overall survival of RCC patients. Older age (> 50 years) was strongly associated with higher risk of RCC development and decreased bikunin mRNA expression.

To investigate the probable mutations of *bikunin*, the 4 exonic regions were examined by SSCP and nucleotide sequence analysis. Only 4th exon of bikunin [10th exon of AMBP (Alpha1-microglobulin/bikunin precursor)] showed a formerly identified polymorphism rs80057939. A > G substitution was observed in 13 tumor tissues (26%) of RCC patients. However, it was not statistically significant ($p > 0.05$). There is no significant relationship between RCC pathogenesis and nucleotide sequence of bikunin suggesting that somatic mutations may not be a cause of bikunin inactivation.

Cancer remains a significant health burden worldwide. As a potential approach to the RCC treatment, gene constructs or exogenous administration of bikunin might be of clinical use due to possessing significant clinical impact, low production cost and minimum side effects [38]. As it is not antigenic to human, it has been suggested as a drug for patients with tumor [21], cerebral ischemia [39], and severe acute pancreatitis [40]. Therefore, a drug such as bikunin that manipulate signal transduction on the plasminogen activator (PA) system in malignant cells may offer a new potential approach to anti-cancer therapy. Bikunin treatment may be beneficial as an adjuvant therapy against metastasis in patients with RCC.

Additionally, a recent study indicated the elevated urinary α1-microglobulin level is associated with a range of important clinical outcomes [41]. Alpha1-microglobulin was a urinary biomarker associated with incident renal cancer [41]. However, in this report the molecular cause of increased α1-microglobulin level was not discussed in detail. As known, alpha1-microglobulin and bikunin are products of a cleavage of one polypeptide chain [42]. They have different functions, however. The study of O'Seaghdha and coworkers [41] is one of the few studies indicating the role of α1-microglobulin in cancer. Interestingly, Medetognon-Benissan and coworkers observed a positive correlation between the urinary excretion of bikunin and α1-microglobulin in healthy controls, while there was no such correlation in CaOx stone formers [43]. However, these two studies were performed measuring the protein level. The inconsistency between the results of our study and O'Seaghdha *et al*. requires further protein analysis. The molecular mechanisms regulating the *bikunin* expression, the up- and down-stream events were not identified, as well.

According to our data, bikunin is a good marker for poorer prognosis in patients with RCC, especially in clear cell subtype. Expression of bikunin gene is significantly decreased among patients who are older than fifty years (see Table 2) and the risk for RCC is higher among the same group of patients, as well (see Table 3). Therefore, bikunin mRNA expression in RCC might be used as a prognostic marker. However, these are preliminary results and need to be verified by determination of protein levels in larger populations.

In conclusion, a statistically significant relationship was established between the reduced level of bikunin and RCC pathogenesis compared to normal kidney tissue samples. To date, the regulation of bikunin expression, the downstream events of bikunin signaling, and all of the genes that response to bikunin remain mostly unclear. Future studies investigating the regulation of bikunin improve our understanding of the biological significance of bikunin in healthy and cancer tissues, clearly.

ACKNOWLEDGEMENTS

We would like to thank to Assoc. Prof. Seval Kul, Head of the Biostatistics Department, for her help in statistical evaluations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. **Kiyama R, Zhu Y, Aoyagi T.** Genetics of renal tumors. In: Renal Tumor. J Chen, Ed. Tokyo: Intech, 2013.

2. **Yin-Goen Q, Dale J, Yang WL, *et al.*** Advances in molecular classification of renal neoplasms. Histol Histopathol 2006; **21**: 325–39.

3. **Kobayashi H, Suzuki K, Sugino D, *et al.*** Urinary trypsin inhibitor levels in amniotic fluid of normal human pregnancy: decreased levels observed at parturition. Am J Obstet Gynecol 1999; **180**: 141–7.

4. **Pugia MJ, Valdes R Jr, Jortani SA.** Bikunin (urinary trypsin inhibitor): structure, biological relevance, and measurement. Adv Clin Chem 2007; **44**: 223–45.

5. **Salier JP, Chan P, Raguenez G, *et al.*** Developmentally regulated transcription of the four liver-specific genes for inter-alpha-inhibitor family in mouse. Biochem J 1993; **296**: 85–91.

6. **Itoh H, Tomita M, Kobayashi T, *et al.*** Expression of Inter-alpha-trypsin inhibitor light chain (bikunin) in human pancreas. J Biochem 1996; **120**: 271–5.

7. **Daveau M, Jean L, Soury E, *et al.*** Hepatic and extrahepatic transcription of inter-alpha-inhibitor family genes under normal or acute inflammatory conditions in rat. Arch Biochem Biophys 1998; **350**: 315–23.

8. **Businaro R, Leali FM, De Renzis G, *et al.*** Inter-alpha-trypsin inhibitor-related immunoreactivity in human tissues and body fluids. Cell Mol Biol 1992; **38**: 463–71.

9. **Shikimi T, Hattori K, Takaori S.** Existence of a human urinary trypsin inhibitor (urinastatin)-like substance in the rat brain. Jpn J Pharmacol 1992; **60**: 97–103.

10. **Yoshida E, Sumi H, Maruyama M, *et al.*** Distribution of acid stable trypsin inhibitor immunoreactivity in normal and malignant human tissues. Cancer 1989; **64**: 860–9.

11. **Fries E, Blom AM.** Bikunin — not just a plasma proteinase inhibitor. Int J Biochem Cell Biol 2000; **32**: 125–37.

12. **Shigetomi H, Onogi A, Kajiwara H, *et al.*** Anti-inflammatory actions of serine protease inhibitors containing the Kunitz domain. Inflamm Res 2010; **59**: 679–87.

13. **Reuning U, Magdolen V, Wilhelm O, *et al.*** Multifunctional potential of the plasminogen activation system in tumor invasion and metastasis (review). Int J Oncol 1998; **13**: 893–906.

14. **Suzuki M, Kobayashi H, Tanaka Y, *et al.*** Suppression of invasion and peritoneal carcinomatosis of ovarian cancer cell line by overexpression of bikunin. Int J Cancer 2003; **104**: 289–302.

15. **Kobayashi H.** Suppression of urokinase expression and tumor metastasis by bikunin overexpression [mini-review]. Hum Cell 2001; **14**: 233–6.

16. **Kobayashi H, Fujie M, Shinohara H, *et al.*** Effects of urinary trypsin inhibitor on the invasion of reconstituted basement membranes by ovarian cancer cells. Int J Cancer 1994; **57**: 378–84.

17. **Kobayashi H, Suzuki M, Kanayama N, *et al.*** Suppression of urokinase receptor expression by bikunin is associated with inhibition of upstream targets of extracellular signal-regulated kinase-dependent cascade. Eur J Biochem 2002; **269**: 3945–57.

18. **Kobayashi H, Suzuki M, Tanaka Y, *et al.*** 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 2001; **276**: 2015–22.

19. **Amin MB, Amin MB, Tamboli P, *et al.*** Prognostic impact of histologic subtyping of adult renal epithelial neoplasms: an experience of 405 cases. Am J Surg Pathol 2002; **26**: 281–91.

20. **Truong LD, Shen SS.** Immunohistochemical diagnosis of renal neoplasms. Arch Pathol Lab Med 2011; **135**: 92–109.

21. **Kobayashi H, Suzuki M, Hirashima Y, *et al.*** The protease inhibitor bikunin, a novel anti-metastatic agent. Biol Chem 2003; **384**: 749–54.

22. **Delaria KA, Muller DK, Marlor CW, *et al.*** Characterization of placental bikunin, a novel human serine protease inhibitor. J Biol Chem 1997; **272**: 12209–14.

23. **Wilharm E, Parry MA, Friebel R, *et al.*** Generation of catalytically active granzyme K from Escherichia coli inclusion bodies and identification of efficient granzyme K inhibitors in human plasma. J Biol Chem 1999; **274**: 27331–7.

24. **Baker MS, Bleakley P, Woodrow GC, *et al.*** Inhibition of cancer cell urokinase plasminogen activator by its specific inhibitor PAI-2 and subsequent effects on extracellular matrix degradation. Cancer Res 1990; **50**: 4676–84.

25. **Duffy MJ.** The urokinase plasminogen activator system: role in malignancy. Curr Pharm Des 2004; **10**: 39–49.

26. **Danø K, Behrendt N, Høyer-Hansen G, *et al.*** Plasminogen activation and cancer. Thromb Haemost 2005; **93**: 676–81.

27. **Price JT, Bonovich MT, Kohn EC.** The biochemistry of cancer dissemination. Crit Rev Biochem Mol Biol 1997; **32**: 175–253.

28. **Blood CH, Zetter BR.** Tumor interactions with the vasculature: angiogenesis and tumor metastasis. Biochim Biophys Acta 1990; **1032**: 89–118.

29. **Duffy MJ.** The biochemistry of metastasis. Adv Clin Chem 1996; **32**: 135–66.

30. **Brown PD.** Clinical studies with matrix metalloproteinase inhibitors. APMIS 1999; **107**: 174–80.

31. **Deryugina EI, Quigley JP.** Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev 2006; **25**: 9–34.

32. **Brooks SA, Lomax-Browne HJ, Carter TM, *et al.*** Molecular interactions in cancer cell metastasis. Acta Histochem 2010; **112**: 3–25.

33. **Suzuki M, Kobayashi H, Tanaka Y, *et al.*** Bikunin target genes in ovarian cancer cells identified by microarray analysis. J Biol Chem 2003; **278**: 14640–6.

34. **Kobayashi H, Suzuki M, Kanayama N, *et al.*** Genetic down-regulation of phosphoinositide 3-kinase by bikunin correlates with suppression of invasion and metastasis in human ovarian cancer HRA cells. J Biol Chem 2004; **279**: 6371–9.

35. **Hamm A, Veeck J, Bektas N, *et al.*** Frequent expression loss of Inter-alpha-trypsin inhibitor heavy chain (ITIH) genes in multiple human solid tumors: a systematic expression analysis. BMC Cancer 2008; **8**: 25.

36. **Igci M, Arslan A, Igci YZ, *et al.*** Bikunin and alpha1-microglobulin/bikunin precursor (AMBP) gene mutational screening in patients with kidney stones: a case-control study. Scand J Urol Nephrol 2010; **44**: 413–9.

37. **Pugia MJ, Lott JA.** Pathophysiology and diagnostic value of urinary trypsin inhibitors. Clin Chem Lab Med 2005; **43**: 1–16.

38. **Gou XH, Liu YY, Chen QL, *et al.*** High level expression of bikunin in Pichia pastoris by fusion of human serum albumin. AMB Express 2012; **2**: 14.

39. **Yano T, Anraku S, Nakayama R, Ushijima K.** Neuroprotective effect of urinary trypsin inhibitor against focal cerebral ischemia-reperfusion injury in rats. Anesthesiology 2003; **98**: 465–73.

40. **Xia Q, Yuan L, Yang XN, *et al.*** Comparison of integrated Chinese and Western medicine with and without somatostatin supplement in the treatment of severe acute pancreatitis. World J Gastroenterol 2005; **11**: 1073–6.

41. **O'Seaghdha CM, Hwang SJ, Larson MG, *et al.*** Analysis of a urinary biomarker panel for incident kidney disease and clinical outcomes. J Am Soc Nephrol 2013; **24**: 1880–8.

42. **Pierzchalski P, Rokita H, Koj A, *et al.*** Synthesis of alpha 1-microglobulin in cultured rat hepatocytes is stimulated by interleukin-6, leukemia inhibitory factor, dexamethasone and retinoic acid. FEBS Lett 1992; **298**: 165–8.

43. **Médétognon-Benissan J, Tardivel S, Hennequin C, *et al.*** Inhibitory effect of bikunin on calcium oxalate crystallization *in vitro* and urinary bikunin decrease in renal stone formers. Urol Res 1999; **27**: 69–75.