

**Aim of the study:** We measured the impact of changing KLK6 expression levels on the pathological grade of gliomas and on proliferation rate, cell cycle progression, and apoptosis in the U251 glioblastoma cell line.

**Material and methods:** The expression of KLK6 in 35 brain glioma tissues and adjacent noncancerous tissues was measured using real-time quantitative polymerase chain reaction (PCR) and the relationship between KLK6 expression and pathological grades was analysed.

**Results:** The KLK6 expression in U251 cells was silenced by a specific siRNA, and the effects on proliferation, the cell cycle, and apoptosis were compared to wild type cells. Expression of KLK6 was downregulated in gliomas relative to matched noncancerous tissue. There was no obvious relationship between patient sex, pathological grade, or tumour classification and the expression of KLK6. In the U251 cell line, cell proliferation was enhanced and the fractions of cells in the G2 and S phases were increased by siRNA-mediated KLK6 silencing.

**Conclusions:** Expression of KLK6 inhibits tumour growth. Decreased KLK6 expression may be a possible risk factor for glioma.

**Key words:** KLK6, kallikrein, U251, siRNA, gliomas.

*Contemp Oncol (Pozn)* 2014; 18 (4): 246–251  
DOI: 10.5114/wo.2014.44628

# Correlation between KLK6 expression and the clinicopathological features of glioma

Jiangtao Lou\*, Haiyan Si\*, Yingjian Chen, Xiaoming Sun, Hua Zhang, Aijun Niu, Chengjin Hu

\*These authors contributed equally to this work.

Department of Laboratory Medicine, PLA General Hospital of Jinan Military Command, Shandong, PR China

## Introduction

Kallikreins (KLK1-15) are a subgroup of the serine protease family of enzymes. Many studies have shown that kallikreins are aberrantly expressed in many types of cancer, such as prostate, breast, and ovarian cancer [1]. High kallikrein expression has been associated with both poor and favourable patient prognosis [2, 3].

Elevated expression of the human kallikrein 6 gene (KLK6), encoding human kallikrein 6 protein (hK6), was associated with poor prognosis in ovarian cancer and colorectal cancer [4, 5], suggesting that KLK6 may promote tumour progression. Other reports, however, found that KLK6 was downregulated in renal cell carcinoma and salivary gland tumours [6, 7], but there is still no direct evidence that KLK6 inhibits tumour progression.

Glioma is characterised by high invasion, migration, and proliferation abilities. Our limited understanding of the aetiology and growth regulation of gliomas has hindered the development of adjuvant therapies. Kallikrein 6 was shown to be highly expressed in the central nervous system and involved in several neurodegenerative diseases [8, 9]. To date, however, only one study has investigated the relationship between KLK6 and gliomas. Immunohistochemical staining revealed that the KLK6 was downregulated in malignant gliomas compared to benign gliomas [10]. The prognostic value of kallikrein 6 was not analysed, but the possibility of an inhibitory role in tumour growth warrants further study [10].

## Material and methods

### Patients

Thirty-five patients (16 male and 19 female) with primary glioma were treated surgically at the Department of Neurosurgery, General Hospital of Jinan Command between November 2009 and September 2010. This work was performed according to the guidelines of Jinan General Hospital of PLA, which abides by the Helsinki Declaration on ethical principles for medical research involving human subjects. All subjects gave informed consent to this work. Fresh surgical samples, including eight adjacent brain tissues from glioma patients, were immediately snap-frozen in liquid nitrogen. The age of patients ranged from 17 to 70 years (median 45 years). The tumour diagnoses were confirmed by histopathology according to the 2000 World Health Organization (WHO) criteria [11]. No patients had received preoperative radiation, chemotherapy, or immunotherapy treatments.

## Cell culture

The U251 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) supplemented with 10% foetal bovine serum (FBS; Hyclone, USA), 100 IU/ml penicillin, and 100 mg/ml streptomycin. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The medium was changed once every three days. Cells were harvested by trypsinisation and reseeded 24 hours prior to transfection on 24-well plates at a density of 20,000 cells/well in 0.5 ml DMEM with 10% FBS, or at 4000 cells/well in 100 µl DMEM plus 10% FBS on 96-well plates. Cells were transfected at 30–50% confluence.

## KLK6 gene silencing

One short interfering dsRNA for KLK6 was designed and synthesised by Ribobio Co., Ltd (Guangzhou, China): sense 5'-GUGCUGGGGAUGAGAAGUAdTdT-3', anti-sense 3'-dTdTACGACCCCUACUCUUCAU-5'. A control siRNA exhibiting no significant sequence similarity to the human, mouse, or rat *KLK6* gene sequence served as a negative control (Ribobio Co., Ltd). Transfection of siRNA was performed with Lipofectamine 2000 transfection reagent (Invitrogen, USA) following the manufacturer's protocol. Transfected cells were grown at 37°C for 6 hours, followed by incubation with complete DMEM. Cells were analysed after 24 hours by real-time quantitative polymerase chain reaction (RT-PCR) and CCK-8 assay. After 48 hours they were analysed by flow cytometry (FCM), and after 72–84 hours by immunocytochemistry. All experiments included three treatment groups: a blank control group (untreated U251 cells), a negative control group transfected with the random siRNA, and a group transfected with the KLK6-specific siRNA. All experiments were performed in triplicate.

## RNA isolation and RT-PCR

Real-time quantitative PCR (qRT-PCR) was performed to compare the expression level of KLK6 in glioma tissue and adjacent tissues and between the three U251 cell lines. Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions, and reverse transcribed using the PrimScript™ RT reagent Kit (TaKaRa, Japan) to generate cDNAs. Both KLK6 and GAPDH were amplified using specific primer pairs. The primers used to amplify human KLK6 were synthesised according to (NM\_002774) in Genbank: forward primer, 5'-TTCCTGCAGGGTGATTCT-3' and reverse primer, 5'-CACTTGGCCTGAATGGTTTT-3'. The GAPDH gene (internal control) was amplified using the primer pair forward 5'-GAAGGTGAA-GGTCCGAGTC-3' and reverse 5'-GAAGATGGTGATGGGATTC-3'. Real-time PCR was performed using SYBR Premix Ex™ Taq (TaKaRa, Japan). According to manufacturer's recommendation, the cycle conditions were one pre-incubation step at 95°C for 5 minutes, followed by 40 cycles of amplification at 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds, and finally generation of a melting curve at 95°C for 15 seconds, 60°C for 30 seconds, and increase the temperature from 60°C to 95°C at a rate of 0.1°C/sec with continuous measurements of the SYBR Green I signal intensities. The relative expression level was

determined by  $2^{-Ct(\text{GAPDH})-Ct(\text{KLK6})}$ . The PCR products were analysed by 1.5% agarose gel electrophoresis, ethidium bromide staining, and visualisation under UV illumination.

## Immunocytochemistry

For immunocytochemical staining of cultured cells, 20,000 cells/well in 0.5 ml DMEM with 10% FBS were plated on coverslips within 24-well plates. At 72 hours after transfection, coverslips were fixed with 95% ethanol for 30 minutes and then washed three times with PBS. The fixed cells were then incubated in primary antibody against HK6 (1 : 400 dilution, Abnova, USA) overnight at 4°C. Immunolabelling was detected with Elivision™ Plus Polymer HRP (Mouse/Goat) immunocytochemistry kits (Maixin Bio, Fuzhou, China) according to the manufacturer's protocol. Cells were counterstained with haematoxylin. Negative controls without primary antibody were run for each experiment. We defined a positive sample as one in which more than one third of cells were stained brown.

The intensity of immunocytochemical staining for hK6 was scored as 0 (no staining), 1 (weak), 2 (moderate), or 3 (strong). In addition, the percentage of cell labelling was graded as less than 25% (negative) or greater than or equal to 25% (positive).

## WST-8 assay

Cell proliferation rate and viability were determined by the WST-8 assay (Dojindo, Japan). Cells (4 × 10 cells per well) were seeded in 96-well microtitre plates (Nunc, Denmark). An aliquot WST-8 (10 µl) was added to each well at 0, 12, 24, 36, and 48 hours after transfection. The plates were incubated for 30 minutes at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>, and the absorbance was read at 450 nm on a MODEL semiautomatic microwell plate reader (BIOPRD, USA).

## Cell apoptosis analysis

Apoptotic cell death was assessed by Annexin-V FITC and propidium iodide (PI) double staining to discriminate apoptotic cells from healthy cells. In brief, after transfection or control incubation, cells were trypsinised and concentrated by mild centrifugation. The cell pellet was washed three times with cold PBS and fixed with cold 75% ethanol at 4°C overnight. Then the cells were washed once with PBS and treated with RNase at 37°C for 30 minutes. Cells of each group were stained according to the instructions of the Annexin-V FITC Apoptosis Kit (Invitrogen, USA), and analysed using a flow cytometer (Beckman Coulter, USA). The data are presented as dot plots showing fluorescence intensity of Annexin-V FITC and PI as analysed by the CXP software (Beckman Coulter, USA).

## Cell cycle analysis

The effects of KLK6 expression on the cell cycle were also studied by flow cytometry. Cells were plated in 24-well sterile plastic plates at 4 × 10 cells/well. At 48 hours after transfection, cells were collected by trypsinisation, washed with PBS, and fixed in 70% ethanol for 30 minutes at 4°C.

The fixed cells were treated with 50 µg/ml RNase A (Sigma-Aldrich) and stained with 50 µg/ml propidium iodide for 20 minutes at 4°C without light before flow cytometric analyses. Analysis was performed using a flow cytometer (Beckman Coulter Elite; Beckman Coulter, Fullerton, CA, USA), and data analysis was performed with Muticycle for Windows 32-bit software (Beckman Coulter, USA).

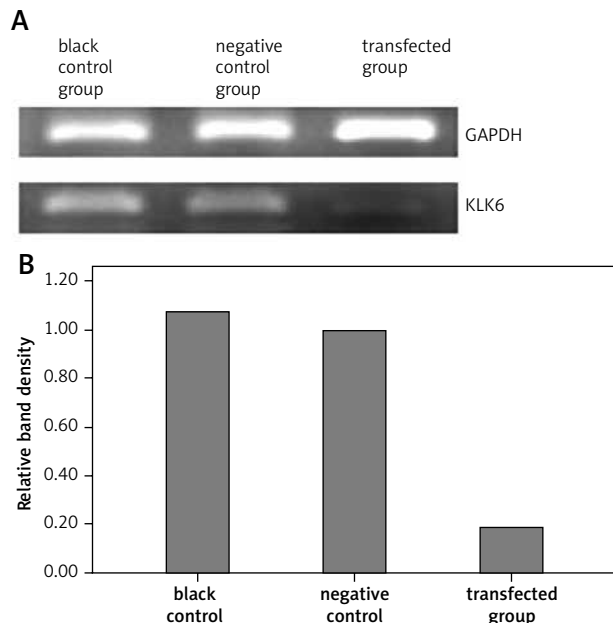
### Statistical analysis

Data are expressed as means ± SD of at least three independent experiments. Differences between the treatment

**Table 1.** Association of KLK6 expression with clinicopathological parameters of glioma

Parameters	No. of cases	mRNA relative expression	P-value
Histological type			< 0.05 <sup>a</sup>
Glioma tissues	8	-3.49 ±1.47	
Adjacent normal brain tissues	8	-2.86 ±1.58	
Glioma tissues classification			NS <sup>b</sup>
Sex			
Male	16	-2.91 ±1.76	
Female	19	-2.65 ±1.52	
Tumour type			NS <sup>b</sup>
Astrocytoma	31	-2.81 ±1.49	
Others	4	-2.45 ±2.61	
Tumour grade			NS <sup>b</sup>
I and II	18	-2.33 ±1.78	
III and IV	17	-3.24 ±1.31	

NS – not significant, <sup>a</sup>Paired-Sample T Test, <sup>b</sup>Independent-Sample T Test



**Fig. 1.** mRNA relative expression level and inhibition ratio. The U251 cell line was transfected with siRNA to target KLK6 or with control siRNA. **A)** Cell lysates were generated 48 h post-transfection. The result showed a significant decrease in KLK6 mRNA level. **B)** Densitometry analysis revealed that U251 cells transfected with siRNA showed a 81.14% decrease compared to negative control group in mRNA level

groups and human tissues were assessed using Student's *t*-tests, and WST-8 assay was analysed by ANOVA test using SPSS 13.0 for Windows (SPSS Inc, USA). *P* values less than 0.05 were considered statistically significant.

## Results

### Expression of KLK6 mRNA in glioma tissues and its association with clinicopathological parameters

Real-time PCR showed that KLK6 expression was down-regulated in gliomas tissue compared to adjacent nontumorous tissue ( $p < 0.05$ ). The expression of KLK6 was not significantly correlated to patient sex, pathological grade, or tumour classification ( $p > 0.05$ ) (Table 1).

### Expression of KLK6 mRNA and hK6 silenced by siRNA

After 48-hour transfection with KLK6-specific siRNA, the expression of KLK6 mRNA was 81.14% lower than the same expression in the negative control group (Fig. 1). Immunocytochemistry also revealed a significant decrease in hK6 protein expression in the KLK6-specific siRNA transfection group (Fig. 2).

### Viability of U251 cells

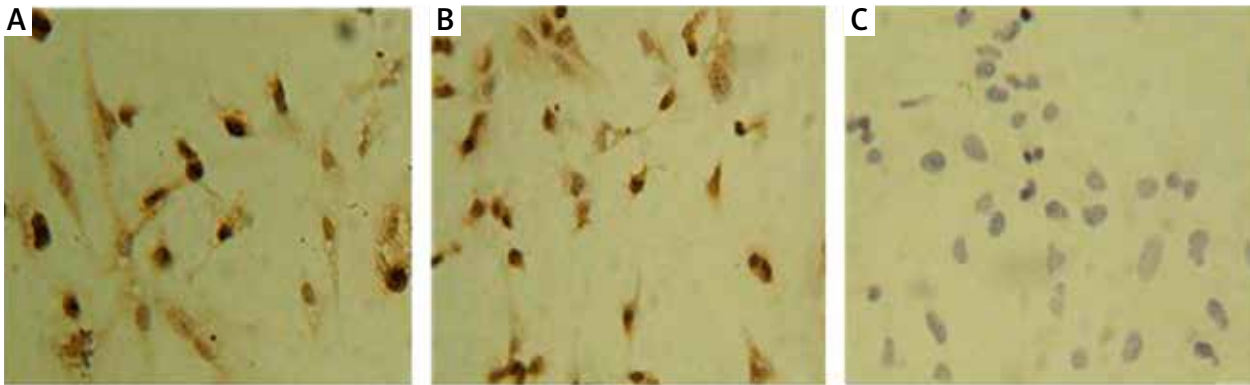
We determined whether inhibition of KLK6 expression in U251 cells inhibited the proliferation by using the WST-8 assay. The total number of viable cells was significantly higher in the KLK6-specific siRNA transfection group, compared to both the blank and negative control groups (Fig. 3), while there was no significant difference between the blank and negative control groups ( $p > 0.05$ ).

### Cell cycle

The possible effect of KLK6 on U251 cell cycle progression was assessed in blank controls, negative controls, using the KLK6 siRNA-transfected cultures. The percentages of cells in G1, S, and G2 phases in blank control group cells (G1: 62.25 ±1.34, S: 24.57 ±1.11, G2: 13.18 ±1.50) and the negative control group (G1: 64.31 ±1.18, S: 23.91 ±0.12, G2: 11.77 ±1.12) were significantly different from the transfected group (G1: 48.45 ±0.77, S: 34.64 ±0.72, G2: 16.91 ±1.47). Knockdown of the *KLK6* gene was associated with a significant increase in the proportion of cells in S and G2 phase (Fig. 4). Compared with negative control group, the percentages of transfected cells in S and G2 phases were increased by 10.72% ( $p < 0.01$ ) and 5.14% ( $p < 0.05$ ).

### Silencing of KLK6 induces apoptosis

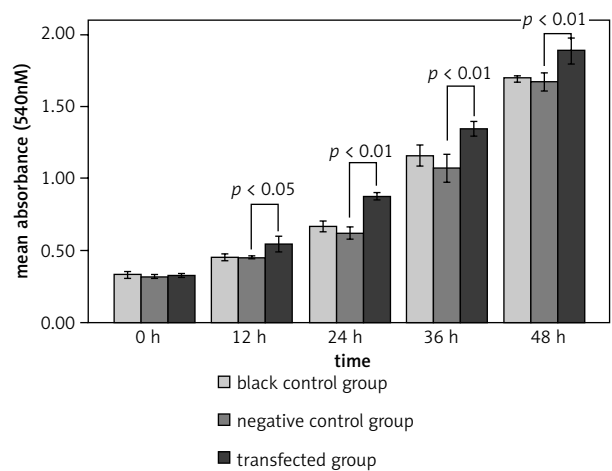
Cells treated with KLK6 siRNA (50 nM) or control siRNA (50 nM) for 48 hours were evaluated for apoptosis frequency by flow cytometry. The percentage of apoptotic cells was examined by Annexin-V and propidium iodide (PI) double staining. The percentages of apoptotic cells in the three groups were not significantly different (Fig. 5), indicating that suppression of KLK6 mRNA expression does not increase apoptosis.



**Fig. 2.** Expression of hk6 in u251 cell line silenced by siRNA. Immunohistochemical stain of U251 cell with monoclonal antibody against hk6. **A)** Blank control group; high-intensity signal was scored as '3'. **B)** Negative control group; high-intensity signal was scored as '3'. **C)** Transfected group; low-intensity signal was scored as '1'

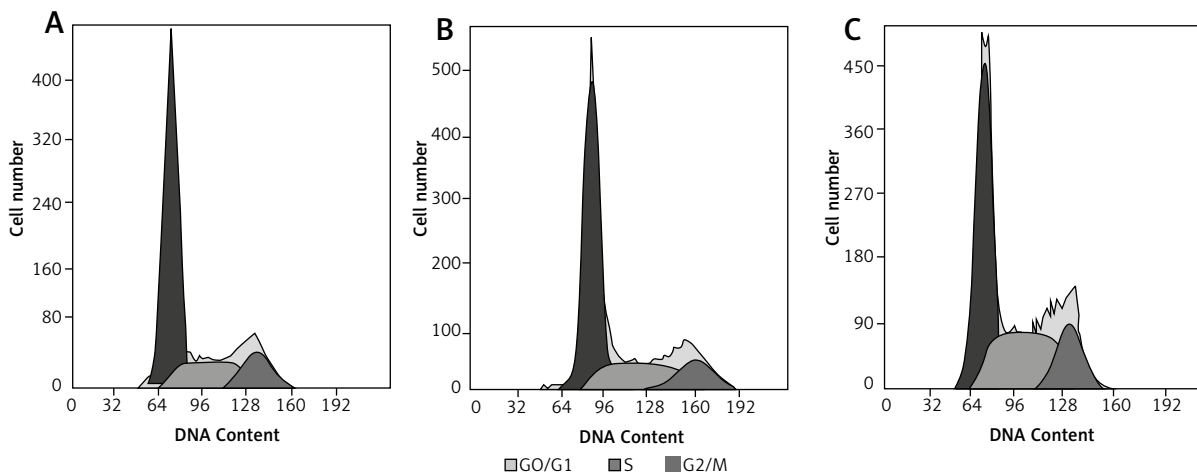
**Discussion**

The human kallikrein-related peptidase (KLK) family consists of 15 highly conserved serine proteases encoded by the largest uninterrupted cluster of protease genes in the human genome. Some members of the family have been reported to be potential cancer biomarkers. Many studies [12–17] have linked the overexpression of human kallikreins (hKs) in cancer to a poor clinical prognosis, but the finding that increased levels of some hKs predict a favourable outcome suggests that these proteins may act as anti-tumour proteases in some tissues. The KLK3 protein might suppress tumour growth and inhibit cell migration and adhesion by activating transforming growth factor  $\beta$  (TGF- $\beta$ ) and protease-activated receptor (PAR) signalling [12, 13]. Furthermore, KLK3 also possesses anti-metastatic properties that are probably derived from its antiangiogenic activity [14]. The expression of KLK8 confers a favourable clinical prognosis to patients with non-small-cell lung cancer [15], while KLK10-transfected breast cancer cells show reduced proliferative activity and diminished potential to generate tumours in nude mice [16]. The KLK14 isozyme can also act as a tumour suppressor, probably by generating angiostatic factors [17]. Here we demonstrate that KLK6 appears to inhibit carcinogenesis, consistent with previous studies showing that it can directly catalyse the

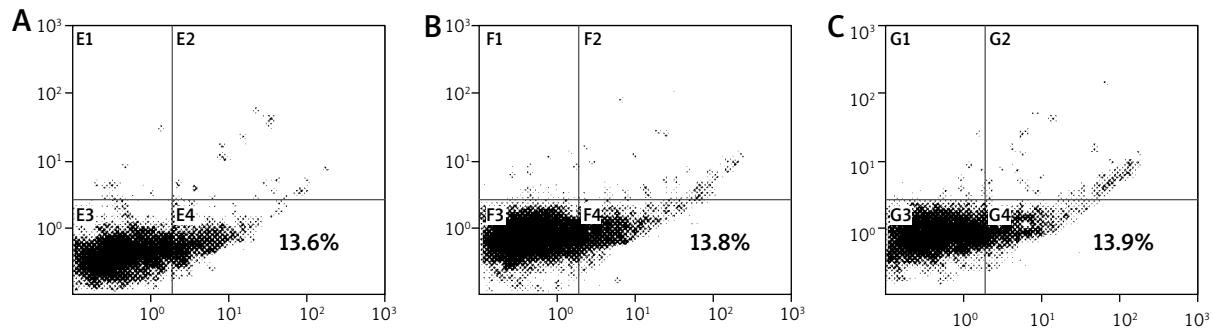


**Fig. 3.** WST-8 assays after treatment with specific siRNA for the KLK6 genes were performed to assess the role of the expression of KLK6 in cancer cell proliferation

hydrolysis of several ECM proteins that facilitate tumour invasion and metastasis [18]. Indeed, KLK6 was downregulated in glioma tissue from eight paired samples of glioma tissues and corresponding non-glioma tissue. Our result, therefore, does not support the immunohistochemical results of Strojnik *et al.* [10], possibly due to the small sample size. Furthermore, we found that suppression of the KLK6



**Fig. 4.** Cell cycle analysis silenced by siRNA. **A)** blank control group, **B)** negative control group, **C)** transfected group



**Fig. 5.** Silencing of KLK6 induces apoptosis in U251 cells

gene could increase the cell numbers in S and G2/M phase and enhance the proliferative activity of a glioma cell line. Thus, our study suggests that KLK6 plays a protective role in glioma patients, and that high expression of KLK6 inhibits proliferation of tumour cells. While conflicting results suggest that KLK6 may have different functions in different transformed cells types, our results provide preliminary support for an antitumor function of KLK6 in glioma.

In addition to tumours, KLK6 expression was increased in the allosum and ischaemic region following occlusion of the middle cerebral artery in mice [19]. The content of KLK6 and hk6 was increased 2 to 7 days following local hypothermic injury [20]. In addition, a decreased CSF concentration of hk6 may be a risk factor for developing Alzheimer's disease [21], and others have found a significantly decreased CSF concentration of hk6 in frontotemporal dementia [22]. These studies suggested that the expression of KLK6 might have a broad protective function in multiple pathological states. A stable and optimal level of KLK6 may be necessary for normal function, while up- or downregulation may lead to distinct pathological conditions. Other studies, however, have found a reciprocal relationship in some diseases; for example, its expression appears to contribute to the pathogenesis of Parkinson's disease and promote neurodegeneration [23, 24].

In conclusion, our RT-PCR results show that the expression of KLK6 was downregulated in glioma tissue. *In vitro* results revealed that the viability and mitotic activity of the U251 cell line was increased by siRNA-mediated KLK6 suppression. These results provided further evidence that KLK6 plays an inhibitory role in the generation and progression of glioma. The signal transduction pathways remain unclear and require further study.

*The authors declare no conflict of interest.*

## References

- Avgeris M, Mavridis K, Scorilas A. Kallikrein-related peptidases in prostate, breast, and ovarian cancers: from pathobiology to clinical relevance. *Biol Chem* 2012; 393: 301-17.
- Borgoño CA, Michael IP, Diamandis EP. Human tissue kallikreins: physiologic roles and applications in cancer. *Mol Cancer Res* 2004; 2: 257-80.
- Emami N, Diamandis EP. Utility of kallikrein-related peptidases (KLKs) as cancer biomarkers. *Clin Chem* 2008; 54: 1600-7.
- Shan SJ, Scorilas A, Katsaros D, Diamandis EP. Transcriptional up-regulation of human tissue kallikrein 6 in ovarian cancer: clinical and mechanistic aspects. *Br J Cancer* 2007; 96: 362-72.
- Ogawa K, Utsunomiya T, Mimori K, Tanaka F, Inoue H, Nagahara H, Murayama S, Mori M. Clinical significance of human kallikrein gene 6 messenger RNA expression in colorectal cancer. *Clin Cancer Res* 2005; 11: 2889-93.
- Petraki CD, Gregorakis AK, Vaslamatzis MM, Papanastasiou PA, Yousef GM, Levesque MA, Diamandis EP. Prognostic implications of the immunohistochemical expression of human kallikreins 5, 6, 10 and 11 in renal cell carcinoma. *Tumour Biol* 2006; 27: 1-7.
- Darling MR, Jackson-Boeters L, Daley TD, Diamandis EP. Human kallikrein 6 expression in salivary gland tumors. *J Histochem Cytochem* 2006; 54: 337-42.
- Shaw JL, Diamandis EP. Distribution of 15 human kallikreins in tissues and biological fluids. *Clin Chem* 2007; 53: 1423-32.
- Yousef GM, Kishi T, Diamandis EP. Role of kallikrein enzymes in the central nervous system. *Clin Chim Acta* 2003; 329: 1-8.
- Strojnik T, Kavalar R, Zajc I, Diamandis EP, Oikonomopoulou K, Lah TT. Prognostic impact of CD68 and kallikrein 6 in human glioma. *Anticancer Res* 2009; 29: 3269-79.
- Radner H, Blümcke I, Reifenberger G, Wiestler OD. The new WHO classification of tumors of the nervous system 2000. *Pathology and genetics*. *Pathologe* 2002; 23: 260-83.
- Killian CS, Corral DA, Kawinski E, Constantine RI. Mitogenic response of osteoblast cells to prostate-specific antigen suggests an activation of latent TGF-beta and a proteolytic modulation of cell adhesion receptors. *Biochem Biophys Res Commun* 1993; 192: 940-7.
- Lai LC, Erbas H, Lennard TW, Peaston RT. Prostate-specific antigen in breast cyst fluid: possible role of prostate-specific antigen in hormone-dependent breast cancer. *Int J Cancer* 1996; 66: 743-6.
- Fortier AH, Holaday JW, Liang H, et al. Recombinant prostate specific antigen inhibits angiogenesis in vitro and in vivo. *Prostate* 2003; 56: 212-9.
- Sher YP, Chou CC, Chou RH, et al. Human kallikrein 8 protease confers a favorable clinical outcome in non-small cell lung cancer by suppressing tumor cell invasiveness. *Cancer Res* 2006; 66: 11763-70.
- Goyal J, Smith KM, Cowan JM, Wazer DE, Lee SW, Band V. The role for NES1 serine protease as a novel tumor suppressor. *Cancer Res* 1998; 58: 4782-6.
- Borgoño CA, Michael IP, Shaw JL, et al. Expression and functional characterization of the cancer-related serine protease, human tissue kallikrein 14. *J Biol Chem* 2007; 282: 2405-22.
- Magklara A, Mellati AA, Wasney GA, Little SP, Sotiropoulou G, Becker GW, Diamandis EP. Characterization of the enzymatic activity of human kallikrein 6: Autoactivation, substrate specificity, and regulation by inhibitors. *Biochem Biophys Res Commun* 2003; 307: 948-55.
- Uchida A, Oka Y, Aoyama M, et al. Expression of myelencephalon-specific protease in transient middle cerebral artery occlusion model of rat brain. *Brain Res Mol Brain Res* 2004; 126: 129-36.
- Oka Y, Uchida A, Aoyama M, et al. Expression of myelencephalon-specific protease after cryogenic lesioning of the rat parietal cortex. *J Neurotrauma* 2005; 22: 501-10.

21. Mitsui S, Okui A, Uemura H, Mizuno T, Yamada T, Yamamura Y, Yamaguchi N. Decreased cerebrospinal fluid levels of neurosin (KLK6), an aging-related protease, as a possible new risk factor for Alzheimer's disease. *Ann N Y Acad Sci* 2002; 977: 216-23.
22. Menendez-Gonzalez M, Castro-Santos P, Suarez A, Calatayud MT, Perez-Pinera P, Martinez M, Ribacoba R, Gutierrez C. Value of measuring plasmatic levels of neurosin in the diagnosis of Alzheimer's disease. *J Alzheimers Dis* 2008; 14: 59-67.
23. Iwata A, Maruyama M, Akagi T, Hashikawa T, Kanazawa I, Tsuji S, Nukina N. Alpha-synuclein degradation by serine protease neurosin: implication for pathogenesis of synucleinopathies. *Hum Mol Genet* 2003; 12: 2625-35.
24. Scarisbrick IA, Linbo R, Vandell AG, et al. Kallikreins are associated with secondary progressive multiple sclerosis and promote neurodegeneration. *Biol Chem* 2008; 389: 739-45.

**Address for correspondence**

**Chengjin Hu**, PhD  
Department of Laboratory Medicine  
PLA General Hospital of Jinan Military Command  
No. 25 Shifan Road, Tianqiao District, Jinan 250031  
Shandong, China  
e-mail: hcj6289@163.com

**Submitted:** 22.12.2013

**Accepted:** 27.12.2013