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Original Article

Up-regulation of Legumain correlates with tumor progression and poor prognosis in urothelial carcinoma of bladder

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Abstract: Objective: Legumain, a novel asparaginyl endopeptidase, has been shown to be frequently up-regulated in a variety of malignancies and act as a potential tumor oncogene. However, its correlations with the clinicopathological features of urothelial carcinoma of the bladder (UCB) have not yet been elucidated. Thus, the purpose of this study was to investigate the diagnostic and prognostic values of Legumain in this disease. Methods: Real-time quantitative PCR (qRT-PCR) and Western blot were employed to measure Legumain mRNA and protein level. Immunohistochemical (ICH) analysis was performed to detect the expression of Legumain in UCB tissues. The correlation between Legumain expression and clinicopathological features was further analyzed. Results: qRT-PCR and Western blot found that the mRNA and protein expression levels of Legumain were significantly elevated in UCB tissues. ICH results showed that Legumain was mainly expressed in the cytoplasm, and its expression level in UCB samples was significantly higher than that in adjacent non-tumor tissues. Further analysis showed that increased Legumain expression in UCB was associated with advanced tumor stage and lymph node metastasis ($P<0.05$). Kaplan-Meier analysis showed that the UCB patients with a high Legumain expression had shorter overall survival than those with a low Legumain expression. Multivariate analysis demonstrated that Legumain expression was an independent prognostic factor for UCB patients. Conclusions: Our results suggested that the upregulation of Legumain was significantly correlated with tumor progression and might be a potential biomarker for prognosis of bladder cancer patients.

Keywords: Urothelial carcinoma of the bladder, Legumain, prognosis

Introduction

Bladder cancer is the fourth most common cancer in men and the eighth most common cancer in women in the world [1]. Urothelial carcinoma of the bladder (UCB) is the most common histological subtype of bladder cancer and accounts for 90%-95% of cases [2]. When initially diagnosed, bladder cancer in approximately 75% cases is confined to the mucosa or submucosa, which is grouped as non-muscle-invasive bladder cancer, while approximately 25% of bladder cancer patients are diagnosed as muscle-invasive bladder cancer [3]. Despite significant advances in accurate and effective diagnostic and therapeutic methods, bladder cancer remains a highly prevalent and lethal malignancy [4]. Therefore, there are ongoing efforts to identify new biomarkers to improve the diagnosis and prognosis assessment of bladder cancer.

Legumain is an asparaginyl endopeptidase belonging to peptidase family C13 and exhibits high specificity for hydrolysis of asparaginyl bonds [5]. Legumain has been proposed to activate the zymogene progelatinase A. This activated form plays an important role in the degradation of extracellular matrix [6]. Emerging evidence indicated that Legumain was highly expressed in several types of human solid tumors, while it was absent or presented at a

Table 1. Association between Legumain expression and clinicopathological features of bladder cancer

Variable	Group	Total	Legumain expression		*P* value
			Low	High	
Gender	Male	56	22	34	0.949
	Female	30	12	18	
Age (years)	<65	33	14	19	0.665
	≥65	53	20	33	
Histological grade	Low grade	24	12	12	0.217
	High grade	62	22	40	
Tumor size (cm)	<2.5 cm	38	17	21	0.380
	≥2.5 cm	48	17	31	
Tumor stage	<T1	36	27	9	0.000
	≥T1	50	7	43	
Tumor multiplicity	Unifocal	28	13	15	0.364
	Multifocal	58	21	37	
Lymph nodes metastasis	No	67	32	35	0.003
	Yes	19	2	17	

very low level in normal tissues from which the tumors arose [7]. For example, Liu et al showed that tumor cells overexpressing Legumain possessed increased migratory and invasive activity in vitro and adopted an invasive and metastatic phenotype in vivo, inferring significance of Legumain in tumor invasion and metastasis [8]. Ohno et al suggested that Legumain might contribute to the invasiveness and aggressiveness of prostate cancer [9]. Wang et al found that a sensitive assay for early expression of Legumain may serve as both a potential biomarker and a molecular target for treatment of ovarian cancer [10]. Those studies supported the view that Legumain plays a significant role in tumor progression. However, the correlation between Legumain expression and clinicopathologic features or prognosis of human bladder cancer has not been reported until now. Thus, the aim of this study was to determine the clinical significance and prognostic values of Legumain in bladder cancer.

Materials and methods

Patients and specimens

A total of 86 formalin fixed, paraffin embedded tissues of UCBs diagnosed between January 2009 and December 2010 at the Department of Urology, Shanghai Tenth People's Hospital of Tongji University (Shanghai, China) was retrieved. For qRT-PCR and Western blot analysis, we collected 30 paired fresh UCBs and normal tissue samples from patient's who underwent surgery between January 2014 and December 2014. None of the patients recruited in this study received preoperative treatment, such as radiotherapy or chemotherapy. The diagnoses were confirmed by pathological findings. The disease stage of each patient was classified or reclassified according to the 2002 AJCC staging system [11]. Written informed consent was obtained from each patient, and research protocols were approved by the Ethical Committee of Shanghai Tenth People's Hospital of Tongji University. Clinicopathological features in our study are presented in **Table 1**.

Real-time quantitative PCR

Total RNA was extracted from tissues using TRIzol reagent (Invitrogen) according to the description of the product. M-MLV Reverse Transcriptase system (Promega) was used for cDNA synthesis. Specific primers for human β-actin were 5'-GGCATCCACGAAACTACCTT-3' (sense primer) and 5'-CTCGTCATACTCCTGCTTGC-3' (anti-sense primer). Specific primers for human Legumain were 5'-GCAGGTTCAAATGGCTGGTAT-3' (sense primer) and 5'-GGAGTGGGATTGTCTTCAGAGT-3' (anti-sense primer). The PCR amplification were performed for 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, on a Applied Biosystems 7900HT (Applied Biosystems) with 1.0 μl of cDNA and SYBR Green Real-time PCR Master Mix (Takara). Data was collected and analyzed by SDS2.3 Software (Applied Biosystems). The relative quantification of gene expression was analyzed with the $2^{-\Delta\Delta Ct}$ method. Each experiment was repeated at least three times.

Western blot

Total proteins from tissues were separated with 10% SDS polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Bioscience). Membranes were blocked for 1 h in TBS-Tween-20 containing 5% non-fat milk and then incubated with anti-Legumain primary

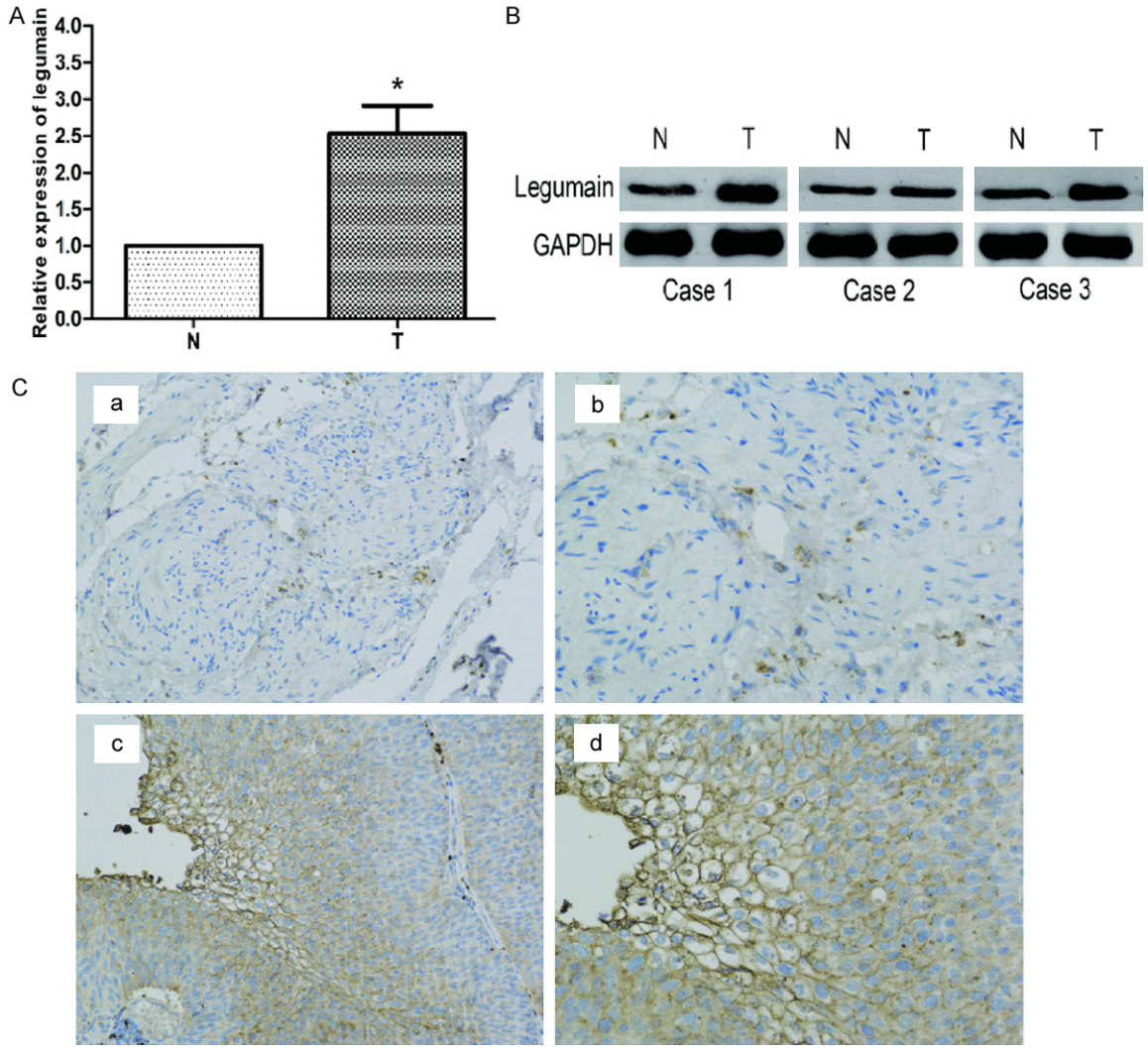

Figure 1. The expression of Legumain in UCB and adjacent non-tumor tissues. A. Expression of Legumain mRNA in UCB tissues and adjacent non-tumor tissues tested by qRT-PCR. B. Expression of Legumain validated by Western blot analysis of three UCB tissues and adjacent non-tumor tissues (T, tumor tissues; N, adjacent non-tumor tissues). C. ICH staining of Legumain in paraffin-embedded human UCB tissues and adjacnet non-tumor tissues. (a) ICH staining of Legumain in adjacent non-tumor tissues (×100). (b) ICH staining of Legumain in adjacent non-tumor tissues (×200). (c) ICH staining of Legumain in UCB tissues (×100). (d) ICH staining of Legumain in UCB tissues (×200). *P<0.05.

antibody (1:200 dilution, Abcam) at 4°C overnight. After being washed 3 times, the blots were incubated with HRP-linked secondary antibodies at room temperature for 1 h. The blots were analyzed by SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) according to the manufacturer's instruction. All experiments were repeated at least three times.

Immunohistochemistry

All samples were fixed in 10% formaldehyde solution, embedded in paraffin blocks, cut in 4 μm thick sections, and mounted on glass slides. Each slide was dewaxed in xylene and rehydrated in grade alcohol, followed by boiling in 10 mmol/L of citrate buffer (PH 6.0) for antigen retrieval. After inhibition of endogenous peroxidase activities for 30 minutes with methanol containing 0.3% H_2O_2, the sections were blocked with 2% bovine serum albumin for 30 minutes and incubated overnight at 4°C with primary anti-Legumain antibody (Abcam). After washing thrice with PBS, the slides were incubated with horseradish peroxidase-conjugated mouse-anti-rabbit IgG for 30 minutes, followed

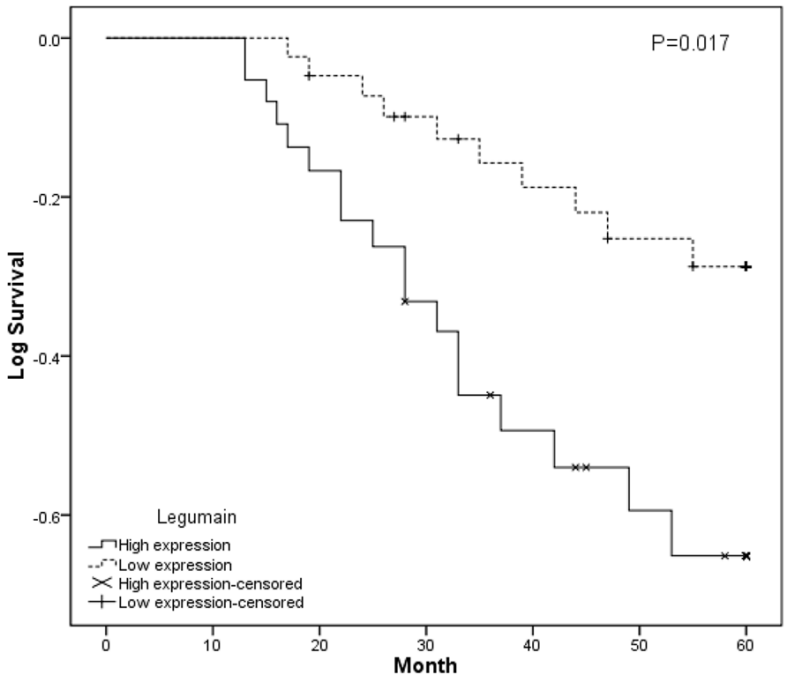

Figure 2. Kaplan-Meier survival curves for UCB patients according to the expression of Legumain.

by reaction with diamin obenzidine and counterstaining with Mayer/hematoxylin. Negative control was done by omission of the primary antibody and substituting it with nonspecific rabbit IgG.

The positive staining of Legumain in the tissue was observed under microscope, and the percentage of bladder tumor cells with Legumain-positive staining was calculated. Legumain expression level in tissues was determined by the staining index with scores of 0 (0), 1 (0 to <5%), 2 (5 to <25%), 3 (25 to <50%), 4 (50 to <75%), and 5 (>75%). A staining index score of ≤2 was defined as low Legumain expression, and staining index score ≥3 as high Legumain expression [10].

Statistical analysis

Statistical analysis of study was performed using SPSS version 18.0 for windows. The significance of differences in Legumain expression between UCBs and adjacent non-tumor tissues was established by Fisher's exact test. A relationship between Legumain expression and clinicopathological features was examined with the χ^2 method or Fisher's exact test. The correlation between Legumain expression and patients' survival was tested by the Kaplan-Meier method. Differences were considered significant if the *P*-value from a two-tailed test was <0.05.

Results

Legumain expression in human UCBs tissues

Legumain expression was first evaluated by qRT-PCR and Western blot. Selected frozen UCB tissues and adjacent non-tumor tissues were used for qRT-PCR assay. Our data showed that Legumain mRNA was significantly increased in UCB tissues compared to that in adjacent non-tumor tissues (**Figure 1A**; P<0.05). Furthermore, Western blot analysis was performed to explored Legumain protein expression in UCB tissues and adjacent non-tumor tissues, our data confirmed that Legumain was significantly increased in UCB tissues compared to that in adjacent non-tumor tissues (**Figure 1B**; P< 0.05). These results revealed that Legumain expression was upregulated at both mRNA and protein levels in bladder cancer.

To assess the clinical relevance of Legumain expression, ICH was used to investigate the protein expression of Legumain in UCB tissues. ICH staining showed that the Legumain protein was mainly accumulated in the cytoplasm of malignant cells. And the expression of Legumain in UCB tissues was significantly higher than that in adjacent non-tumor tissues (**Figures 1C**).

Association of Legumain expression with clinicopathological features of UCB patients

Table 1 summarized the association between Legumain expression and clinicopathological features of UCB patients. High Legumain expression was observed to be closely correlated with advanced tumor stage and lymph node metastasis (P<0.05). In contrast, there was no association between Legumain expression and

Table 2. Univariate and multivariate analyses of prognostic factors in bladder cancer patients

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
Gender	1.271	0.528-2.143	0.514			
Age (years)	1.521	0.498-3.134	0.327			
Tumor multiplicity	0.796	0.375-3.852	0.449			
Tumor stage	3.714	1.911-6.271	0.008	2.984	1.639-5.738	0.014
Histological grade	2.173	0.731-4.256	0.071			
Lymph node metastasis	4.173	1.496-9.175	<0.001	3.136	1.358-7.941	0.002
Legumain	2.983	0.891-8.416	0.018	2.508	0.863-7.931	0.023

other clinicopathological features, such as gender, age, tumor size, histological grade and tumor multiplicity (all P>0.05).

Association of Legumain expression with prognosis of UCB patients

The prognostic value of Legumain expression was explored using the Kaplan-Meier method and log-rank test. As shown in **Figure 2**, there was a significant correlation between Legumain expression and overall survival of UCB patients (P=0.017, log-rank test). The overall survival rate of UCB patients with high Legumain expression was significantly lower than that of those patients with low Legumain expression (**Figure 2**). The univariate and multivariate analyses were also performed to identify factors related to patient prognosis. As shown in **Table 2**, the univariate analysis showed that the tumor stage, lymph node metastasis and Legumain expression were significantly related to overall survival of UCB patients (**Table 2**; P<0.05). Moreover, the multivariate regression analysis suggested that the tumor stage, lymph node metastasis and Legumain expression were independent prognostic factors for UCB patients (**Table 2**; P<0.05).

Discussion

Many clinicopathologic factors, including nuclear matrix protein22 (NMP22), inhibitor of apoptosis protein (IAP) and bladder tumor antigen (BTA) have been commonly used in UCB clinics, but their clinical usefulness remains controversial from diagnostic, prognostic, and surveillance points of view [12-14]. With the advent of genomic and proteomic technologies, it has become possible to explore whether novel cancer-related genes can serve as novel molecular markers for predicting the prognosis of UCB Patients.

Legumain, an asparaginyl endopeptidase, is expressed in several human tissues such as the kidney, placenta, liver, spleen, testis, and bone marrow plasma [15, 16]. Legumain plays an important role in antigen presentation, regulation of osteoclast formation, and extracellular matrix remodeling [17]. Recently, Legumain has been shown to be deregulated in many types of cancers. For example, Murthy et al found that Legumain expression in human ovarian cancer was higher than that in normal tissues, and over-expression of Legumain correlated with increased cell migration and invasion of ovarian cancer cells [10]. Wu et al offered the convincing evidence that the increased expression of Legumain was significantly associated with malignant development of breast cancer and may be a novel prognostic marker [18]. Guo et al indicated that Legumain was upregulated in gastric cancer and had considerable potential in identification of the prognosis [19]. However, the clinical significance of Legumain expression in UCB remains unclear.

In the present study, we found that Legumain expression in UCB tissues was significantly higher than that in adjacent non-tumor tissues at both mRNA and protein levels. Then the relationships of the Legumain with various clinical features of UCB were analyzed. Our data showed that high Legumain expression level was associated with advanced tumor stage and lymph node metastases, indicating that Legumain might be involved in the carcinogenesis and metastasis of UCB. Furthermore, Kaplan-Meier analysis with the log-rank test indicated that UCB patients with high Legumain expression showed a shorter overall survival

than those with low Legumain expression. Univariate and multivariate analyses were utilized to evaluate whether the Legumain expression level and various clinicopathological features were independent prognostic factors of UCB patients. Multivariate analysis revealed that Legumain expression level was independent prognostic factors for overall survival of UCB patients, indicating that high Legumain level was a promising non-invasive biomarker for prognosis of patients with UCB. However, the precise molecular mechanisms behind the altered expression of Legumain in UCB and its function are not very clear. Additional studies are needed to more clearly and comprehensively articulate the molecular mechanisms of both the cause and the effects of altered expression of Legumain in the development and/or progression of UCB

In conclusion, we report that Legumain is associated with poor overall survival of UCB patients, indicating its potential prognostic values in this disease. The findings may provide important insights into understanding the progression of bladder cancer.

Disclosure of conflict of interest

None.

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