# Clinical significance of α- and β-Klotho in urothelial carcinoma of the bladder

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**Abstract.** Non-muscle invasive bladder cancer (NMIBC) accounts for ~70% of all bladder cancers. One of the serious clinical issues related to the management of NMIBC is that it has significant potential to progress to muscle invasive bladder cancer (MIBC) after initial treatments. α-Klotho (KLα), originally identified as an anti-aging gene, has recently been reported to have antitumor effects in various malignancies. In contrast, β-Klotho (KLβ) has been reported to have protumoral functions. However, the associations between KLα/KLβ and the biological behavior of urothelial carcinoma remain unclear. In the present study, we evaluated the association between clinicopathological background factors of NMIBC and the expression levels of KLα or KLβ. A high expression level of KLβ, but not KLα, was an independent predictive factor of short progression-free survival for NMIBC. An elevated level of KLβ correlated with a higher incidence of lymphovascular invasion (LVI). We added *in vitro* assays using human bladder cancer cell lines to investigate the role of KLβ. Treatment with exogenous KLβ protein increased the proliferation, migration, transendothelial migration abilities and anchorage-independent growth of the cell lines. In addition, the KLβ concentration in voided urine samples obtained before initial transurethral surgery was quantitated with enzyme-linked immunosorbent assay (ELISA). The urine KLβ concentration was found to be higher in patients with bladder cancer than that in healthy volunteers. Our results suggest that KLβ plays important roles in tumor invasion and progression, and its concentration may be a valuable urine-based marker for the detection of bladder cancer.

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*Abbreviations:* KLα, α-Klotho; KLβ, β-Klotho; TURBT, transurethral resection of bladder tumor; NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer; FGF, fibroblast growth factor; IHC, immunohistochemistry; FBS, fetal bovine serum; CIS, carcinoma *in situ*; LVI, lymphovascular invasion; HCC, hepatocellular carcinoma; IQR, interquartile range; ELISA, enzyme-linked immunosorbent assay

*Key words:* urothelial carcinoma, non-muscle invasive bladder cancer, α-Klotho, β-Klotho, urine marker

## Introduction

Urothelial carcinoma of the bladder is an important health issue worldwide. In the USA, bladder cancer is the fourth most common malignancy in men and the eighth most common in women, and it is estimated that 74,690 patients were diagnosed with bladder cancer and 15,580 patients died from this malignancy in 2014 (1). Approximately 70% of bladder cancers are diagnosed as non-muscle invasive bladder cancer (NMIBC), including stages Ta and T1 (2,3). The standard treatment for NMIBC is transurethral resection of bladder tumor (TURBT) with or without adjuvant intravesical bacillus Calmette-Guerin therapy, or chemotherapy with anthracyclines, mitomycin, or gemcitabine (4). Although Ta bladder cancer is associated with favorable cancer-specific survival, T1 bladder cancer has a significant potential to progress to muscle invasive bladder cancer (MIBC) after initial treatment. One of the most significant issues is that T1 (almost high-grade) bladder cancer can be a lethal disease with varying degrees of aggressiveness and progression (5-8). T1 high-grade bladder cancer progresses to MIBC at a rate of 25-50% (6,7,9). The important goal of the clinical management for T1 high-grade bladder cancer is to prevent progression of the cancer. Therefore, there is an emerging need to discover novel biomarkers that can predict the progression of the cancer accurately and become a clinically available therapeutic target.

The α-Klotho (KLα) gene was identified as an anti-aging gene in 1997 (10). The authors reported that KLα-knockout mice developed a syndrome that resembles ageing conditions, such as a short lifespan, arteriosclerosis, and osteoporosis. Kurosu *et al* revealed that KLα overexpression in mice extended their lifespan at a rate of 20-30% (11). The KLα protein exists in two forms: a membrane and a secreted form. Membrane KLα functions as a co-receptor of fibroblast growth factor (FGF)23 to regulate phosphate homeostasis (12,13). Secreted KLα is a regulator of oxidative stress activity, multiple growth factor receptors, and ion channels (14,15). The β-Klotho (KLβ) gene was identified in 2000 (16), wherein the authors reported that the amino acid sequence was 41.2% identical to that of KLα.

Notably, both KLα and KLβ lack glucosidase catalytic activity, and this characteristic is the reason why both molecules are recognized as a new and distinct protein family within the glycosidase family 1 superfamily. Whereas the role of KLβ is unclear, membrane KLβ is specifically known to be a co-receptor of FGF19 and FGF21, regulating the synthesis of bile acid and energy metabolism (17-19). Recently, attention has been directed toward the association between cancer and KLα/KLβ. In the case of KLα, most of the studies have suggested it to be a tumor suppressor (20-23). Doi *et al* reported that KLα inhibited transforming growth factor-β1 signaling, which induced epithelial-to-mesenchymal transition responses and suppressed cancer metastasis *in vivo* (24). In contrast to KLα, the association between cancers and KLβ expression has not yet been well investigated. In the present study, we focused on the clinical significance of KLα and KLβ, which could be regulators of the cancer progression of urothelial carcinoma of the bladder.

## Materials and methods

*Human samples*. We extracted tissue samples from 155 NMIBC and 6 MIBC patients who had undergone TURBT between April 2004 and March 2013. Patients who had undergone early cystectomy were excluded from the study. The protocol for the research project was approved by the Institutional Review Board for Clinical Studies (Medical Ethics Committee ID: NMU-900), and informed consent was obtained from all the patients.

*Immunohistochemistry*. To examine the expression levels of KLα and KLβ, immunohistochemistry (IHC) was carried out. We used paraffin-embedded tissues obtained from all 161 patients in the study to examine the association between the KLα/KLβ expression levels and clinicopathological variables. The paraffin blocks were cut and placed on SuperFrost Plus Microscope Slides (Thermo Fisher Scientific, Yokohama, Japan). The sections were deparaffinized and antigen retrieval was carried out in citric acid buffer (pH 6.0) in an autoclave. IHC staining was performed with the Histofine ABC kit (Nichirei, Tokyo, Japan). Briefly, slides were treated with 1% hydrogen peroxide in methanol to block endogenous peroxidase activity. The slides were then incubated overnight at 4˚C with anti-KLα antibody (sc-22220, rabbit polyclonal, dilution 1/500) and anti-KLβ antibody (sc-74343, rabbit polyclonal, dilution 1/200) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. The slides were counterstained with hematoxylin, dehydrated, and mounted on a cover slide. We evaluated each slide using IHC scores (IHC score = intensity score + population score; intensity: none, 0; low, 1; intermediate, 2; and high, 3; population: none, 0; 0-25%, 1; 25-50%, 2; 50-75%, 3; and 75-100%, 4). The KLα/KLβ expression was categorized into low or high according to the IHC score as follows: low, IHC score ≤4; high, IHC score 5 or 6.

*Cell lines*. The human urothelial carcinoma cell lines MGH-U3, J82, and UM-UC-3 were used in this study. MGH-U3 was a gift from Dr H. LaRue (Laval University Cancer Research Centre, Quebec, Canada). J82 and UM-UC-3 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cell lines were maintained in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan), supplemented with 10% fetal bovine serum (FBS; JRH, Tokyo, Japan) and 1% penicillin/streptomycin (Thermo Fisher Scientific) in a standard humidified incubator at 37˚C in an atmosphere of 5% $CO_2$.

*Western blot analysis*. Western blot analysis was performed using protein extracted from the cultured cells. All proteins were extracted using the RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA), which contained 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM $Na_3VO_4$, 1 mM phenylmethylsulfonyl fluoride, and 1 $\mu$g/ml leupeptin. Protein concentrations were quantified using Protein Quantification kit-Wide Range (Dojindo, Kumamoto, Japan). Total protein (20 $\mu$g) was diluted with sodium dodecyl sulfate (SDS) loading buffer containing 2.5% β-mercaptoethanol, boiled at 95˚C for 5 min, and electrophoresed on 10% SDS-polyacrylamide gels using a Mini-PROTEAN Tetra electrophoresis cell at 200 V for 35 min. The gels were then transferred onto polyvinylidene difluoride membranes using a semi-dry transfer apparatus (both from Bio-Rad, Hercules, CA, USA) at 15 V for 45 min. After blocking overnight in Tris-buffered saline (pH 7.6) containing 5% skim milk and 0.1% Tween-20, the membranes were incubated for 1 h with the primary anti-KLα rabbit polyclonal antibody (dilution 1/200), anti-KLβ rabbit antibody (dilution 1/200), and anti-actin mouse monoclonal antibody (dilution 1/10,000) as an internal loading control, followed by 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG antibody. The bound secondary antibody was detected using a West Pico Detection kit (Thermo Fisher Scientific). The secondary antibodies (Santa Cruz Biotechnology) were used at 1:5,000, 1:5,000, and 1:10,000 dilutions, respectively.

*Cell proliferation assays*. Recombinant human KLβ was purchased from R&D Systems (Minneapolis, MN, USA). The cell proliferation assay was used to examine the effects of the exogenous KLβ. Each of the cell lines was incubated with two different concentrations of KLβ (10 or 50 ng/ml) for 48 h in serum-free medium at 37˚C in an atmosphere of 5% $CO_2$.

*Matrigel invasion assay and transendothelial migration assay*. We evaluated whether the cell migration ability would increase with exogenous KLβ. At first, we performed the Matrigel invasion assay using BioCoat Matrigel Invasion Chambers (BD Biosciences, Piscataway, NJ, USA). Each cell line was incubated at 37˚C in an atmosphere of 5% $CO_2$ with or without 50 ng/ml of KLβ. After 48 h of incubation, non-invading cells on the upper chambers were removed and the invading cells in the lower chambers were stained with calcein AM (PromoKine, Heidelberg, Germany) and the cells were immediately examined under a fluorescence microscope (Leica DMI 4000B).

We examined whether exogenous KLβ could increase the ability of the cancer cells to invade the endothelial cell layer, using human umbilical vascular endothelial cells (HUVECs; Lonza, Tokyo, Japan). Using a FluoroBlok insert system, the

insert membrane chambers were coated with 30,000 HUVECs on fibronectin (Wako, Osaka, Japan) for HUVEC adhesion and incubated at 37˚C in an atmosphere of 5% $CO_2$. After 8 h of incubation, low-concentrated colcemid (Nacalai Tesque) was added to the insert membrane to inhibit the migration of endothelial cells. Then, each cancer cell line was sprinkled onto the membrane. After a 24-h incubation period, non-invading cells in the upper chambers were removed and images of the invading cells on the membrane of the lower chambers were captured and visualized under a fluorescence microscope. The cells that attached to the bottom of the membrane were stained and examined as aforementioned.

*Soft agar colony formation assay*. To examine for anchorage-independent growth, we performed a soft agar colony formation assay. The base agar layer was prepared using 1.2% agar solution (Difco, Franklin Lakes, NJ, USA) mixed with an equal volume of 2X Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich)/20% FBS in a 24-well culture plate. Cell suspensions (30,000 cells/ml) were prepared and mixed with both the 1.2% agar solution and 2X DMEM/20% FBS in the same manner as described above. Exogenous KLβ (50 ng/ml) or PBS as a control was added into each well and incubation was carried out at 37˚C in an atmosphere of 5% $CO_2$. A week after seeding, the number of growing colonies was counted under a microscope.

*Measurement of secreted KLβ by ELISA*. Voided urine samples from 59 NMIBC patients and 10 MIBC patients were collected prior to surgery and stored at -80˚C. Urine was also obtained from four healthy volunteers as assay controls. All 73 urine samples were thawed just before use and analyzed for KLβ concentration with an enzyme-linked immunosorbent assay (ELISA) kit (Cloud-Clone Corp., Houston, TX, USA), using a Tecan microplate reader (Tecan Systems, Inc., San Jose, CA, USA).

*Statistical analysis*. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). The figures were also constructed using GraphPad Prism 5.0. The comparison between high and low KLβ expression was calculated using the Mann-Whitney U test or the Student's t-test. The survival curve was obtained using the Kaplan-Meier method and compared by the log-rank test for each prognostic variable. A multivariate analysis was performed using the Statistical Package for the Social Sciences, version 19.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered statistically significant.

## Results

*The association of Klotho expression with various clinicopathological variables*. To investigate the association between KLα/KLβ and various clinicopathological variables, we performed IHC analysis of the KLα/KLβ expression levels. Fig. 1A shows representative images of weak, intermediate, and strong expression of KLα (left panels) and KLβ (right panels). KLα expression was higher in NMIBC than that in MIBC (P=0.0061; Fig. 1B, left), whereas the opposite was true for KLβ expression (P=0.0028; Fig. 1B, right). Table I lists the

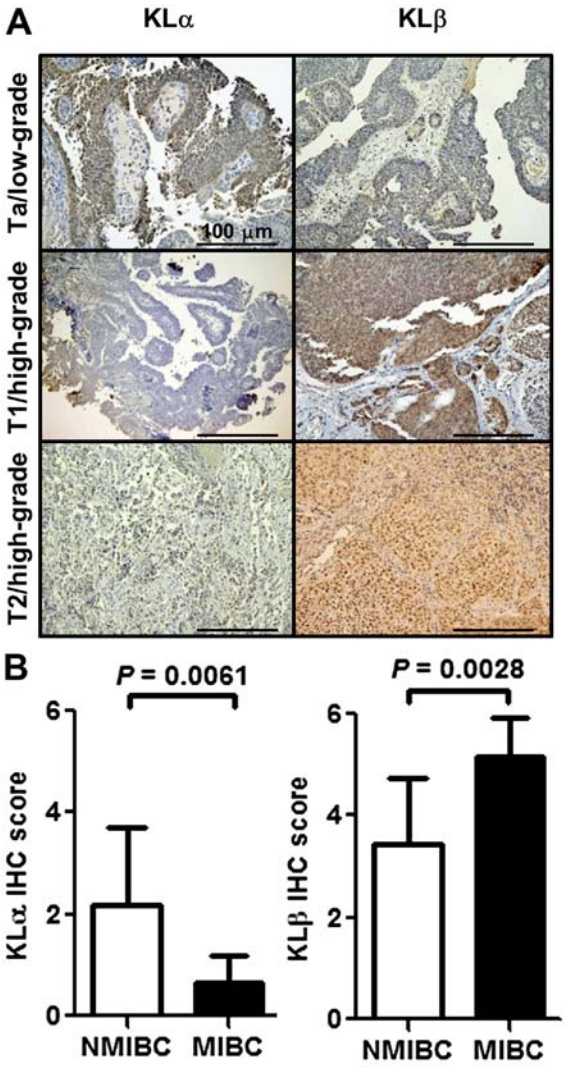


Figure 1. (A) α-Klotho (KLα) was expressed in urothelial carcinoma of the bladder, whereas β-Klotho (KLβ) expression gradually decreased as tumors progressed to a high stage and high grade. KLα and KLβ showed opposite results. Scale bars, 100 μm. (B) Quantification of KLα and KLβ expression levels in non-muscle invasive bladder cancer (NMIBC) compared to muscle invasive bladder cancer (MIBC). KLα expression was significantly higher in NMBIC. On the other hand, KLβ expression was significantly higher in MBIC. IHC, immunohistochemistry.

clinicopathological background of the cohorts of 155 NMIBC patients and comparisons of the variables with high and low KLα/KLβ expression. At initial TURBT, the median age for this cohort was 71 years [interquartile range (IQR) 34-94] and the median follow-up period was 53 months (IQR 1-126). The pathological data, such as the tumor category, tumor grade, and concomitant carcinoma *in situ* (CIS), were significantly different between patients with low and high KLα expression. In the case of KLβ, patients with high expression were significantly of high stage and high grade, and had a high rate of concomitant CIS and high presence of lymphovascular invasion (LVI) compared with patients with low KLβ expression. KLα and KLβ showed opposite trends for both invasiveness and tumor grade.

*The expression level of KLβ, but not KLα, is associated with disease progression to MIBC*. Of the 155 NMIBC patients, 55

Table I. Patient clinicopathological background.

| Variables | No. of patients | KLα expression | | P-value | KLβ expression | | P-value |
|---|---|---|---|---|---|---|---|
| | | Low | High | | Low | High | |
| Total | 155 | 140 | 15 | | 117 | 38 | |
| Gender | | | | 0.67[a] | | | 1.0[a] |
| Male | 138 | 125 | 13 | | 105 | 33 | |
| Female | 17 | 15 | 2 | | 12 | 5 | |
| Age (at initial TURBT) | | | | 0.73[b] | | | 0.19[b] |
| Median (IQR) in years | 71 (34-94) | 71 (36-94) | 72 (34-85) | | 71 (34-94) | 72 (54-89) | |
| Tumor category | | | | 0.0006[a] | | | <0.01[a] |
| Ta | 68 | 56 | 12 | | 66 | 2 | |
| T1 | 73 | 73 | 0 | | 45 | 28 | |
| Tis | 14 | 11 | 3 | | 6 | 8 | |
| Tumor grade | | | | 0.0062[a] | | | <0.0001[a] |
| High | 84 | 81 | 3 | | 48 | 36 | |
| Low | 71 | 59 | 12 | | 69 | 2 | |
| Concomitant CIS with Ta/1 (n=141) | | | | 0.0041[a] | | | 0.0002[a] |
| Yes | 51 | 51 | 0 | | 31 | 20 | |
| No | 90 | 78 | 12 | | 80 | 10 | |
| LVI with T1 (n=73) | | | | - | | | 0.0001[a] |
| Yes | 24 | 49 | 0 | | 7 | 17 | |
| No | 49 | 24 | 0 | | 38 | 11 | |

[a]Chi-square test or Fisher's exact test; [b]Mann-Whitney U test. KLα, α-Klotho; KLβ, β-Klotho; TURBT, transurethral resection of bladder tumor; IQR, interquartile range; CIS, carcinoma *in situ*; LVI, lymphovascular invasion.

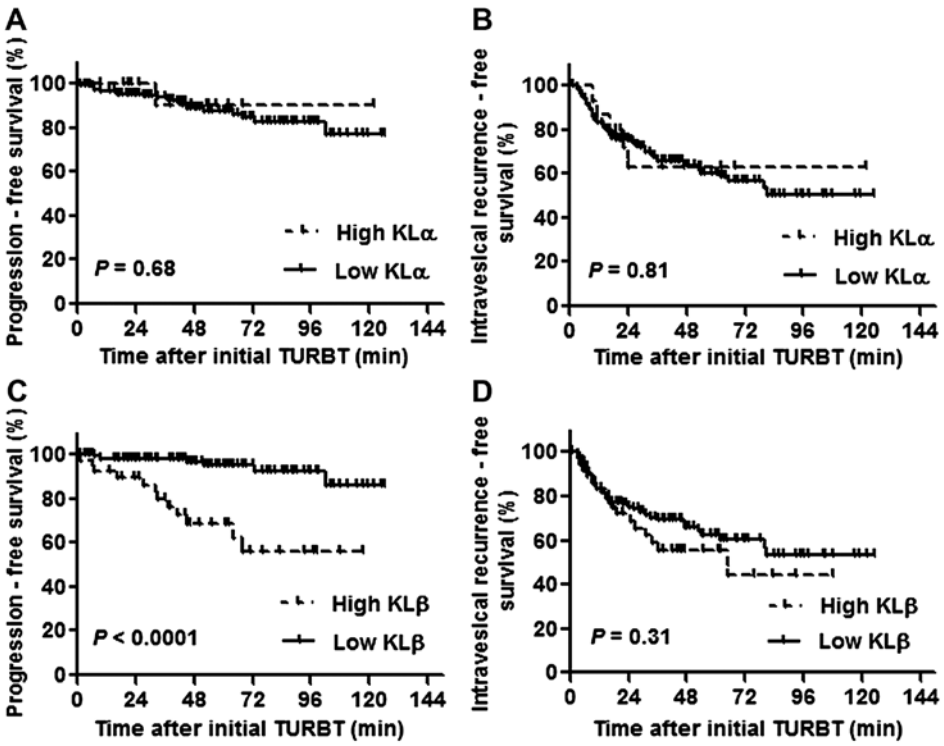


Figure 2. The association between clinicopathological information of the 155 non-muscle invasive bladder cancer (NMIBC) patients and their α-Klotho (KLα)/β-Klotho (KLβ) expression. There was no significant association between KLα expression and both (A) progression-free survival and (B) intravesical recurrence-free survival. (C) On the other hand, the progression-free survival rate was significantly lower in patients with high KLβ expression than that in patients with low KLβ expression. (D) With regards to intravesical recurrence-free survival, there was no significant difference between KLβ expression levels.

Table II. Univariate analysis of prognostic factors for progression- or intravesical recurrence-free survival.

| | Intravesical recurrence-free survival | | | Progression-free survival | | |
|---|---|---|---|---|---|---|
| Variables | HR | 95% CI | P-value | HR | 95% CI | P-value |
| Gender | | | | | | |
| Male | 1 | | | 1 | | |
| Female | 0.99 | 0.39-2.5 | 0.29 | 3.4 | 0.60-19 | 0.17 |
| Tumor category | | | | | | |
| Ta | 1 | | | 1 | | |
| T1 | 0.82 | 0.48-1.4 | 0.5 | 3.8 | 1.1-14 | 0.042 |
| Tis | 0.47 | 0.14-1.6 | 0.22 | 7.1 | 1.6-32 | 0.011 |
| Tumor grade | | | | | | |
| Low | 1 | | | 1 | | |
| High | 0.9 | 0.52-1.6 | 0.73 | 3.5 | 1.4-8.8 | 0.0088 |
| CIS | | | | | | |
| No | 1 | | | 1 | | |
| Yes | 0.92 | 0.54-1.6 | 0.79 | 3.3 | 1.3-8.5 | 0.012 |
| LVI | | | | | | |
| No | 1 | | | 1 | | |
| Yes | 1.8 | 0.82-3.7 | 0.14 | 11 | 3.1-37 | 0.0002 |
| KLβ expression | | | | | | |
| Low | 1 | | | 1 | | |
| High | 1.4 | 0.73-2.6 | 0.3 | 13 | 4.2-38 | <0.0001 |

Chi-square test or Fisher's exact test. HR, hazard ratio; CI, confidence interval; CIS, carcinoma *in situ*; LVI, lymphovascular invasion; KLβ, β-Klotho.

(35.5%) had an intravesical recurrence at median 33.5 months after initial TURBT and 18 patients (11.6%) progressed to muscle invasive tumors at median 51 months after initial TURBT. With regard to the intravesical progression- and recurrence-free survival in patients with high or low KLα expression, there was no significant difference between the two groups [P=0.68 (Fig. 2A) and P=0.81 (Fig. 2B), respectively]. In contrast, the progression-free survival for the patients with high KLβ expression was significantly shorter than that for the patients with low expression (P<0.0001; Fig. 2C). The intravesical recurrence-free survival was not significantly different between the two groups (P=0.31; Fig. 2D).

Table II shows the univariate analysis of prognostic factors for both progression- and intravesical recurrence-free survival. The analysis revealed that high KLβ expression was a poor prognostic factor for progression-free survival [hazards ratio (HR) =13, 95% CI 4.2-38; P<0.0001] but not for intravesical recurrence-free survival (HR=1.4, 95% CI 0.73-2.6; P=0.3). On the other hand, LVI was a predictive factor for progression-free survival (HR=11, 3.1-37; P=0.0002) but not for intravesical recurrence-free survival (HR=1.8, 95% CI 0.82-3.7; P=0.14). The other factors such as T category, tumor grade, and concomitant with CIS showed similar results. Table III shows the multivariate analysis of prognostic factors for progression-free survival. The Cox proportional hazard model analysis identified high KLβ expression (HR=6.9, 95% CI 2.6-18; P<0.0001) as an independent prognostic factor of progression to muscle invasive disease.

Table III. Cox proportional models for prognostic factors.

| | Progression-free survival | | |
|---|---|---|---|
| Variables | HR | 95% CI | P-value |
| Gender | | | |
| Male | 1 | | |
| Female | 2.2 | 0.60-7.7 | 0.24 |
| Tumor category | | | |
| Ta | 1 | | |
| T1 | 0.87 | 0.14-5.4 | 0.89 |
| Tis | 3.3 | 0.58-19 | 0.18 |
| Tumor grade | | | |
| Low | 1 | | |
| High | 0.83 | 0.07-9.7 | 0.88 |
| CIS | | | |
| No | 1 | | |
| Yes | 1.2 | 0.32-4.2 | 0.81 |
| LVI | | | |
| No | 1 | | |
| Yes | 2.3 | 0.78-6.5 | 0.13 |
| KLβ expression | | | |
| Low | 1 | | |
| High | 6.9 | 2.6-18 | <0.0001 |

HR, hazard ratio; CI, confidence interval; CIS, carcinoma *in situ*; LVI, lymphovascular invasion; KLβ, β-Klotho

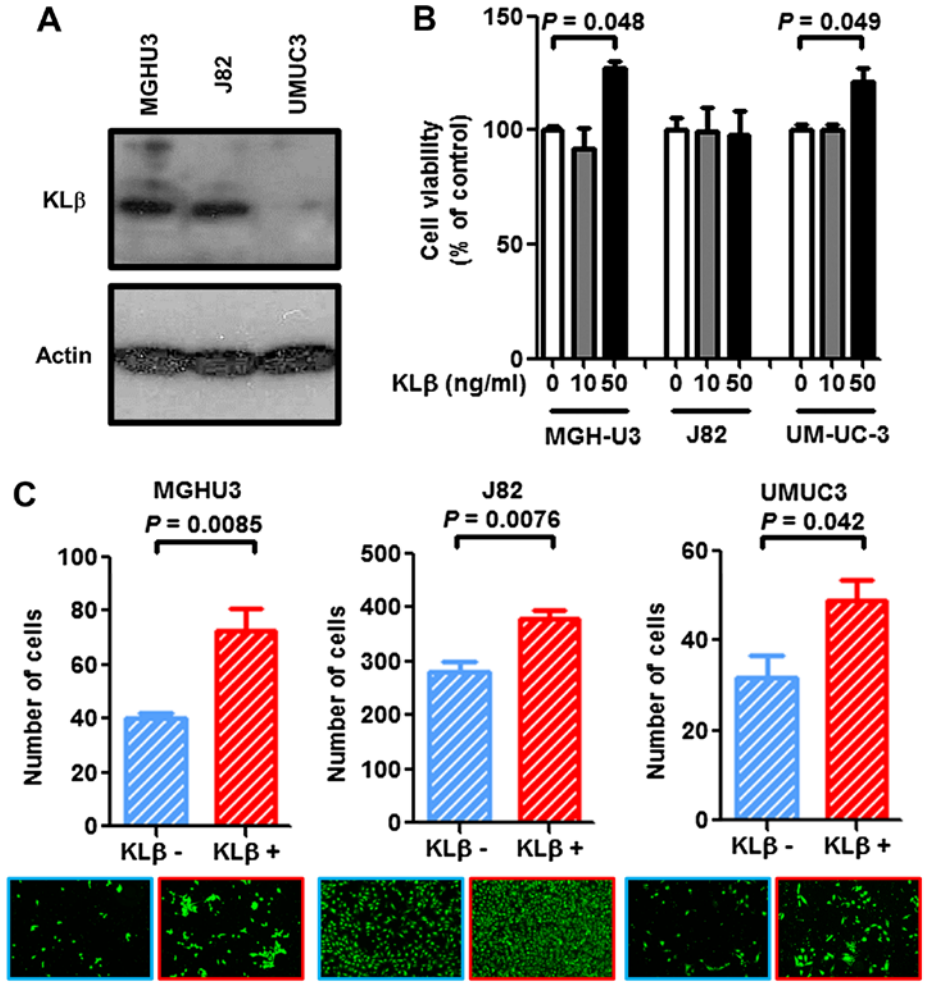


Figure 3. Western blot analysis of β-Klotho (KLβ) in three human urothelial carcinoma cell lines. (A) KLβ expression was decreased in the UM-UC-3 cells. (B) In the proliferation assay, treatment with 50 ng/ml exogenous KLβ increased the proliferation ability of the MGH-U3 and UM-UC-3 cells by ~130%. (C) In the Matrigel invasion assay, treatment with exogenous KLβ increased the migration ability of all three cell lines.

*KLβ promotes the proliferation and tumor invasiveness of urotherial cancer cells*. To verify the expression level of KLβ in the three urothelial cancer cell lines, western blot analysis was performed. There were variations in the endogenous KLβ expression levels (Fig. 3A), where UM-UC-3 expressed the lowest level among the three cell lines. Exogenous KLβ treatment at a concentration of 50 ng/ml promoted urothelial cancer cell proliferation by 120-140% in the MGH-U3 and UM-UC-3 cells, whereas no effect was observed in J82 cells (Fig. 3B). With regard to the Matrigel invasion assay, KLβ treatment enhanced the invasiveness of all three cell lines (Fig. 3C).

*KLβ expression is associated with LVI*. We assessed the association between KLβ expression and LVI status. Tumors with LVI had significantly higher KLβ expression than LVI-negative tumors in the IHC analysis (P<0.0001; Table I and Fig. 4A). To confirm this result with *in vitro* experiments, we used the transendothelial migration assay (Fig. 4B). Cells that broke through the layer of the endothelial cells and migrated to the bottom of the insert were quantified with a fluorescence-based spectrophotometer and microscopy (Fig. 4C). Exogenous KLβ treatment enhanced the transendothelial migration ability of all three cell lines. These findings suggest that KLβ is highly related to disease progression via enhanced tumor invasion through the vessel wall.

*KLβ treatment enhances anchorage-independent growth*. Cells were suspended in soft agar and incubated with or without KLβ. The number of colonies was counted 7 days after seeding. Evaluation on day 7 showed a notable increase in the colony formation ability of cells treated with KLβ in all three cell lines (Fig. 5). The results suggested that stimulation with KLβ enhanced the cell anchorage-independent growth capability *in vitro*.

*Urine KLβ concentration is increased in MIBC patients*. The preoperative voided urine KLβ concentration in MIBC patients was significantly higher than that in NMIBC patients (Fig. 6). However, there was no significant difference between the healthy volunteers and the MIBC patients and NMIBC patients, respectively (Fig. 6). To ascertain the association between urine KLβ concentration and the result of urine cytology, we categorized the studied patients into three groups: negative, class I or II; suspicious, class III; and positive, class IV or V. There were no significant differences in urine KLβ concentration among the groups (data not shown).

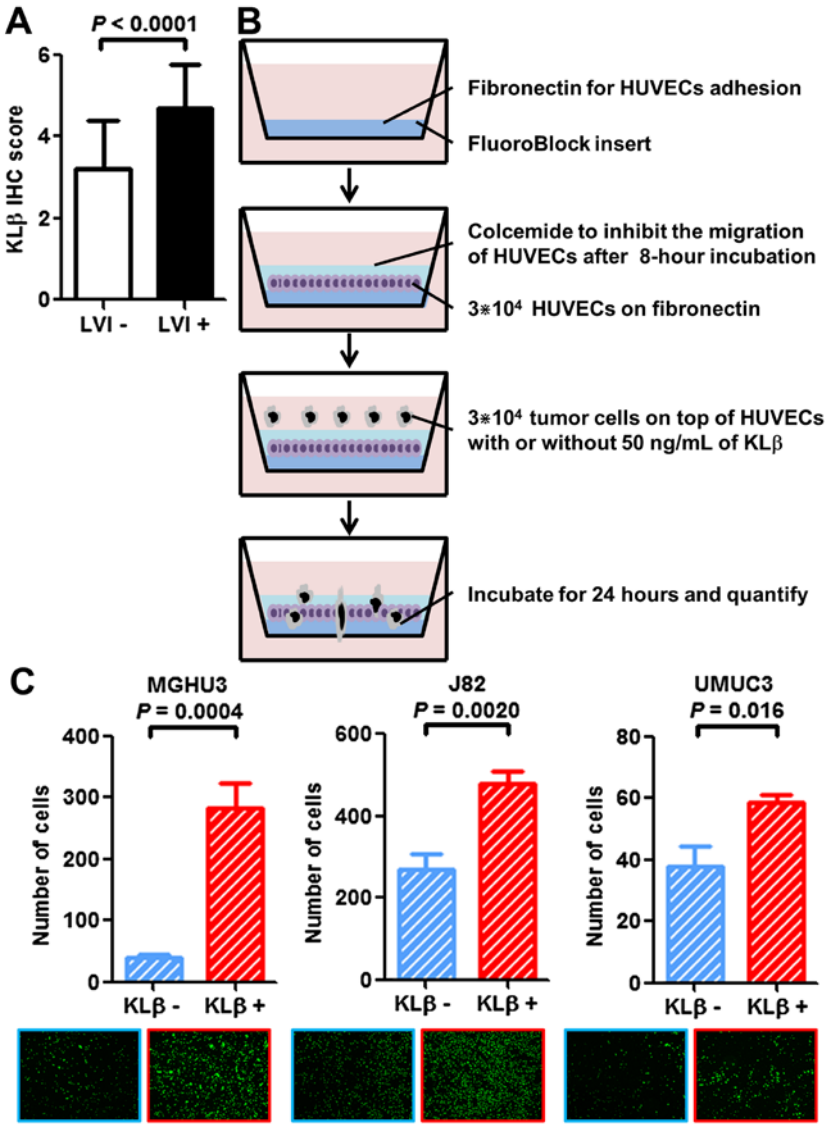


Figure 4. From the clinicopathological information, the progression-free survival for lymphovascular invasion (LVI)-positive patients was significantly lower than for LVI-negative patients. (A) From the immunohistochemistry (IHC) of β-Klotho (KLβ), tumors with LVI had significantly higher KLβ expression than tumors without LVI. (B) To evaluate the effect of exogenous KLβ on transendothelial migration ability, we prepared upper inserts using human umbilical vascular endothelial cells (HUVECs). Each cell line was incubated with or without 50 ng/ml of KLβ for 24 h. (C) The results showed that treatment with exogenous KLβ increased the transendothelial migration ability of all three human urothelial carcinoma cell lines.

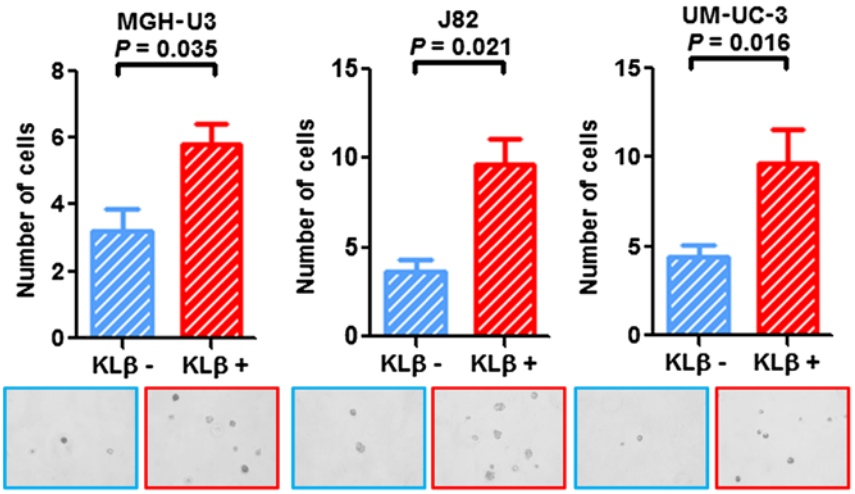


Figure 5. Soft agar assay with or without treatment with exogenous β-Klotho (KLβ) in three human urothelial carcinoma cell lines. The results showed that treatment with exogenous KLβ increased the anchorage-independent growth capability of all three human urothelial carcinoma cell lines.

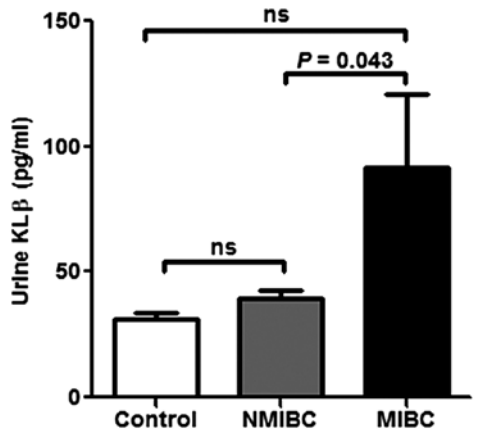


Figure 6. To evaluate whether the urine β-Klotho (KLβ) concentration has potential as a detection marker of urothelial bladder carcinoma, the urine KLβ concentration was quantified by enzyme-linked immunosorbent assay (ELISA). The concentration in muscle invasive bladder cancer (MIBC) patients was significantly higher than those in both healthy volunteers and non-muscle invasive bladder cancer (NMIBC) patients. ns, not significant.

## Discussion

Our present results demonstrated the trend that KLα is overexpressed in low-stage and low-grade tumors whereas KLβ is overexpressed in high-stage and high-grade tumors of human bladder cancer. The multivariate analysis revealed that a high KLβ expression level is an independent prognostic factor for progression to muscle invasive disease. However, KLα expression is associated neither with intravesical recurrence- nor with progression-free survival. Thus, we hypothesized that for urothelial carcinoma of the bladder, KLα acts as a tumor suppressor whereas KLβ acts as a tumor promotor.

In 2008, Wolf *et al* first reported that KLα acted as a tumor suppressor in breast cancer (20). In their IHC study, the KLα expression in normal breast tissue was higher than that in breast cancer cells, and high KLα expression was associated with smaller tumor size in breast cancer samples. In their *in vitro* study, the cDNA or siRNA of KLα was transfected into the MCF-7 cell line (which normally expresses KLα) and resulted in 60% reduction or a 2.5-fold increase in cell growth, respectively. The authors suggested the possibility of KLα having tumor-suppressing roles on cell proliferation and survival, mediated by inhibition of the IGF-1/insulin signaling pathway. Xie *et al* revealed that a decreased level of KLα protein expression and an increased methylation level of the KLα gene promoter region are poor prognostic factors in hepatocellular carcinoma (HCC) (21). In the human lung cancer cell line A549, KLα expression inhibited cancer proliferation and induced apoptosis of the cells (22). In the case of urogenital carcinoma, Zhu *et al* showed that the survival rate for patients with high KLα expression was significantly longer than that for patients with low expression, and KLα suppressed the epithelial-to-mesenchymal transition and migration and invasion of renal cell carcinoma cells (23). In the present study, although KLα expression was significantly higher in NMIBC than in MIBC, KLα was not a favorable prognostic factor of bladder cancer for intravesical recurrence- and progression-free survival. Our IHC study suggested that KLα expression possibly acts as a tumor suppressor in bladder cancer. In order to verify this, we need to increase the number of study cases or to examine a cohort of MIBC patients.

Poh *et al* showed that elevated KLβ expression contributed to HCC progression through the FGF4 signaling pathway (25). On the contrary, Ye *et al* reported the antiproliferative effect of KLβ by regulating the Akt/GSK-3β/cyclin D1 signaling pathway in HCC (26). Therefore, the role of KLβ on tumorigenesis and progression is still controversial. Feng *et al* showed that FGF19 contributed to the promotion of prostate cancer progression and KLβ possibly promoted the pathway aforementioned (27). IHC analysis in the present study showed that KLβ expression was associated with tumor invasiveness and progression. However, we did not examine other factors involved in KLβ, such as FGFs, so we were not able to describe the possible mechanism for the tumor aggressiveness. Similar to the situation in HCC (21), KLβ may regulate the phosphorylation of ERK1/2, FRS2, and Akt, resulting in a progression-promoting cell cycle or the inhibition of cancer cell apoptosis. Further investigation is ongoing to elucidate the detailed mechanisms associated with KLβ, using pathway assays related to FGFs.

KLβ treatment promoted cell proliferation, cell invasiveness, and anchorage-independent growth in the human bladder cancer cell lines. According to a meta-analysis by Kim *et al*, the presence of LVI in TURBT specimens contributed to an increase in the risk of pathological upstaging (28). Moreover, LVI was reported to be an unfavorable prognostic factor for T1 bladder cancer (29). In our study, KLβ treatment increased the invasion ability of all three human bladder cancer cell lines. The IHC examination showed a significant relationship between KLβ expression and LVI, indicating that KLβ stimulates LVI. Evaluation of the anchorage-independent growth in a soft agar assay demonstrated that exogenous KLβ treatment increased the colony formation ability of all the human bladder cancer cell lines studied. The cell cycle or apoptosis is possibly regulated by the direct or indirect influence of KLβ.

The urine KLβ level measured by ELISA was significantly higher in MIBC patients than in NMIBC patients in our study. However, there was no significant difference in urine KLβ levels between bladder cancer patients and healthy controls. Although we expected urine KLβ to be a biomarker for discriminating the malignant potential of bladder cancer, the differential results in the present study did not reach statistical significance. There was a trend of higher urine KLβ levels in patients with bladder cancer than in the controls. However, we need to examine more cases to make concrete conclusions. Notably, the urine KLβ level of the MIBC patients was higher than that of the NMIBC patients. Therefore, we may use pre-TURBT urine KLβ levels to clinically distinguish MIBC from NMIBC, so as not to subject patients to unnecessary examinations before TURBT. Moreover, we can select a case with indication for adjuvant intravesical instillation therapy after TURBT.

In conclusion, we postulate that KLβ acts as a tumor promotor in human bladder cancer, and that the urine KLβ level is a possible biomarker for distinguishing NMIBC from MIBC.

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