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Original Article

High expression of Notch ligand Jagged2 is associated with the metastasis and recurrence in urothelial carcinoma of bladder

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Abstract: Background: The Notch signaling pathway is closely related with human organ development and tumorgenisis. Jagged2 is among the most popular topic in Notch studies currently. Recent studies found its vital role in tumor metastasis in breast cancer; however, its expression profile and its prognostic value in urothelial carcinoma of bladder have not been investigated. Methods: Immunohistochemistry was used to detect the expression of Jagged2 in 120 bladder urothelial carcinoma. Moreover, the expression of Jagged2 was analyzed by Western blot in 60 bladder urothelial carcinoma and 20 normal epithelial tissues. MTT assay and flow cytometry and transwell assay were used to examine the proliferative and invasive ability of bladder cancer cells with the treatment of GSIXX (the inhibitor of Jagged2). Prognostic value of Jagged2 expression and its correlation with tumor metastasis and recurrence were evaluated, and the proliferative and invasive ability and cell cycle process of the bladder cancer cells were detected as well. Results: There was a significantly higher Jagged2 expressions in bladder urothelial carcinoma and highly invasive bladder T24 cells than those in bladder normal tissues and the superficial bladder BIU-87 cells. Jagged2 expression was positively correlated with histological grade, p T stage, recurrence, and metastasis. With the increasing concentration of GSIXX, we found that not only the cell proliferation and invasion activity decreased significantly, but also the cell cycle was blocked at G_2/M stage. Conclusions: Jagged2 expression status was closely correlated with important histopathologic characteristics (grades and stages) and the recurrence and metastasis of bladder urothelial carcinomas. Furthermore, Jagged2 played an important function on the bladder cancer cells' proliferation by regulating the cancer cell cycle from G_1/S to G_2/M and probably promoted the invasion and metastasis of bladder cancer.

Keywords: Bladder urothelial carcinoma, Jagged2, histopathologic grade, clinical stage, GSIXX

Introduction

Bladder cancer is the second most common urologic cancer with relatively high morbidity and mortality [1]. Since it is mostly non-muscle-invasive at initial presentation, up to 70% of patients suffered from fatigue of cancer recurrence and up to 15% will eventually progress to muscle-invasive bladder urothelial carcinoma [2]. Although conventional clinicopathologic parameters such as grade, stage, and lymph node status of the tumor are widely considered be predictors of metastasis, recurrence, and survival, urologists intend to explore novel biomarkers associated with histopathologic features and biological behavior of bladder urothelial carcinoma, which is more efficient for early detection and/or for predicting the metastasis of superficial tumors to invasive higher-stage lesions with higher specificity and sensitivity.

The Notch signaling pathway regulates cell fate decision, proliferation, and death. Emerging evidence suggests that the Notch signaling pathway is likely to be involved in the tumorigenesis. Recent studies demonstrated that Notch signaling plays a critical role in self-renewal of stem cells, and aberrant expression of this

pathway is often observed in various types of cancers [3-5]. However, since the activation of Notch signaling relies on the receptor-ligand interaction, the exact role of peri-tumor microenvironment in Notch activation is not well clarified. Previous studies showed that not only Notch signaling but also Notch ligands were upregulated in high grade cancers, indicating the prognostic value of Notch ligands in clinical settings [6-8]. There are five Notch ligands (Jagged1-2 and Delta like 1, 3 and 4) that have been identified in mammals [9, 10]. They are functionally similar and appear to be redundant; however, expressions of these genes are differentially regulated during embryogenesis as well as in tumor progression. Despite their high sequence similarity, Jagged1 and Jagged2 are differentially regulated according to their distinct biological roles in non-small lung cancer cells [11]. Notably, over-expression of Jagged2 has been found in over 90% of pancreatic cancer cell lines [12]. In breast cancer, increased Jagged2 mRNA was found in the MDA-MB-435 derived Br4 variant cell line which specifically metastasizes to the brain, and knocking down of Jagged2 significantly decreased migration and invasion abilities of the Br4 cells [13]. These evidences suggest that Jagged2 plays an important role in tumor metastasis in breast cancer although the underlying mechanism of the aberrant Jagged2 expression is yet to be defined.

Among mammalian the notch ligands, the oncogenic role of Jagged2 has been emphasized in human triple negative breast cancer. Intriguingly, Jagged2 directly correlates with hypoxia-induced Notch activation and it is essential to promote epithelial-mesenchymal transition (EMT) so that cells at the hypoxic invasive front could survive [14]. The use of Jagged2 inhibitor has increased our knowledge if Jagged2 intracellular regulation and allowed us to make further investigation to suppress tumor growth in vitro. GSIXX, a gamma secretase inhibitor, exhibits anti-proliferative and anti-inflammatory properties in some tumor cells, it can significantly constrain melanoma cell growth and induce apoptosis [15].

In the present study, we observe that Jagged2 has high endogenous expression levels in bladder cancer cell lines and bladder cancer tissues. Overexpression of Jagged2 in tumors closely correlates with a poor prognosis in patients. Furthermore, small molecule inhibitor GSIXX for Jagged2 significantly inhibits bladder cancer cell growth and makes cell cycle arrest. These data suggest that the role of Jagged2 can be important in bladder cancer.

Materials and methods

Patients, recurrence, and follow-up analysis

From 1999 to 2011, at the Department of Urology, Tongji University Affiliated Shanghai Tenth People's Hospital in China, a total of 120 consecutive patients with bladder urothelial cell carcinoma underwent transurethral resection, partial cystectomy, and radical cystectomy. Furthermore, 20 normal urothelial specimens were obtained by biopsy or from cystectomy, which were used as normal controls. The study was conducted according to an institutional review board-approved protocol, and written informed consent was obtained from each patient for surgery and for research purposes. A total of 120 cases were identified, including 100 men and 20 women with a median age of 62 years (range from 35-82 years). The cohort included 76 cases with transurethral resection, 24 cases with partial cystectomy, and 20 cases with radical cystectomy. The tumor size ranged from 0.7 to 10.0 cm in greatest dimension (median size: 3.5 cm). Histologically, the tumors were classified according to the 2004 World Health Organization histologic classification of urinary tract tumors, including 7 papillary urothelial neoplasms of low malignant potential (PUNLMP), 59 low grade papillary urothelial carcinoma, and 54 high grade papillary urothelial carcinoma [16]. Tumors were staged by the American Joint Committee on Cancer system of 2002, including 58 urothelial carcinoma without invasion (<pT1), and 62 invasive urothelial carcinoma (≥pT1) [17]. No patients received adjuvant chemotherapy or radiation therapy before surgery. All patients with noninvasive bladder carcinomas (Ta-T1) were treated with intra-vesical chemotherapy after transurethral resection, whereas all patients with invasive disease (T2-T4) were treated with chemotherapy or radiotherapy after cystectomy. 100 patients with bladder reservation received routine urine examination, chest X-ray, abdominal and pelvic ultrasonography, cystoscopy, and cytology every 3 months. In this series, the recurrence was defined as a new urothelial carcinoma recurring in the blad-

der and/or posterior urethra. During the follow-up period, tumor recurrences and metastasis were observed in 77 and 37 patients, respectively. The median follow-up time was 65 months for patients still alive at the time of analysis. 50 of 120 patients were dead in the follow-up period. Among the 50 patients who died, 36 died of bladder cancer and the remaining 14 died of other causes without evidence of tumor metastasis.

Immunohistochemistry and evaluation

Briefly, formalin-fixed, paraffin-embedded tissue blocks were cut into 4 μm sections and subjected to immunohistochemistry with anti-Jagged2 goat polyclonal antibody (1:200; R&D System, Inc). Immunohistochemistry was carried out using the streptavidin-peroxidase-conjugated method. Negative controls were prepared by substituting PBS substituting for primary antibody. Metastatic breast carcinoma was used as a positive control. Immunohistochemical staining was performed according to previously published methods with minor modification [18]. The sections were incubated with secondary antibodies and visualized using Envision-plus kit (Dako Corp.) and ABC staining system (Santa Cruz Biotechnology). All the immunoreactions were separately evaluated by 2 senior pathologists. The immunoreactivity for Jagged2 was considered positive when brown particles appeared in cytoplasm. The intensity of Jagged2 immunostaining (1=weak, 2=moderate, and 3=intense) and the percentage of positive tumor cells (0%=negative, 1%-50%=1, 51%-75%=2, ≥76%=3) were assessed in at least 5 high power fields (×400 magnification). The scores of each cancerous tissue sample were multiplied to give a final score of 0, 1, 2, 3, 4, 6, or 9, and the tumors were finally determined as negative: score 0; lower expression: score ≤4; or higher expression: score ≥6.

Western blotting

Frozen tissues (including tumor and non-cancerous portion) or cells were washed twice with ice-cold phosphatebuffered saline (PBS), homogenized on ice in 10 volumes (wt/vol) of lysis buffer containing 20 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 1% Triton-X100, 1 mM PMSF, and phosphatase inhibitor using a homogenizer (Heidoph, DLA×900). The homogenate was centrifuged at 12,000 g for 30 minutes at 4°C. The supernatant was collected and stored at -70°C. Protein content was determined by the BCA assay (BCA protein assay kit, Pierce Biotechnology, Rockford, IL). From each sample preparation, 80 μg of total protein was separated by 8% SDS-PAGE and then transferred to PVDF blotting membranes. The total protein extracts were analyzed by immunoblotting with indicated antibodies following SDS-PAGE analysis. Immunoblots were performed using goat polyclonal primary antibodies specific for Jagged2 and mouse monoclonal antibody for α-tubulin (Cell Signaling). After blocking nonspecific binding with 5% BSA in TBS (pH 7.5) containing 0.05% Tween-20 (TBST), primary antibodies were incubated on the membranes for Jagged2 (1:200) and α-tubulin (1:1000) overnight at 4°C in TBST. Following 3 times washes in TBST, the membranes were incubated for 2 hours at 37°C with goat polyclonal secondary antibody to mouse IgG (1:5000, Abcam, Hongkong) labeled with horseradish peroxidase. The proteins were detected using an ECL detection system (Pierce), as directed by the manufacturer. Specific bands for Jagged2 and α-tubulin were identified by prestained protein molecular weight marker (MBI Fermentas, Glen Burnie, MD). The EC3 Imaging System (UVP Inc., Cambridge, UK) was used to catch up the specific bands, and the optical density of each band was measured using Image J software. The ratio between the optical density of Jagged2 and α-tubulin of the same sample was calculated as relative content and expressed graphically.

Cell lines and cultures

Bladder cancer cell lines BIU-87 and T24 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in the culture medium recommended by the American Type Culture Collection and were harvested by using treatment with 0.25% trypsin (Invitrogen, Grand Island, NY) when they were in the logarithmic phase of growth for use in the following experiments.

Cell proliferation assays

T24 cells (1×10^4 cells per well) in logarithmic growth phase were cultured in 96-well flat-bottomed plates in a triplicate pattern and dimethylsulfoxide (DMSO) vehicle alone (control group)

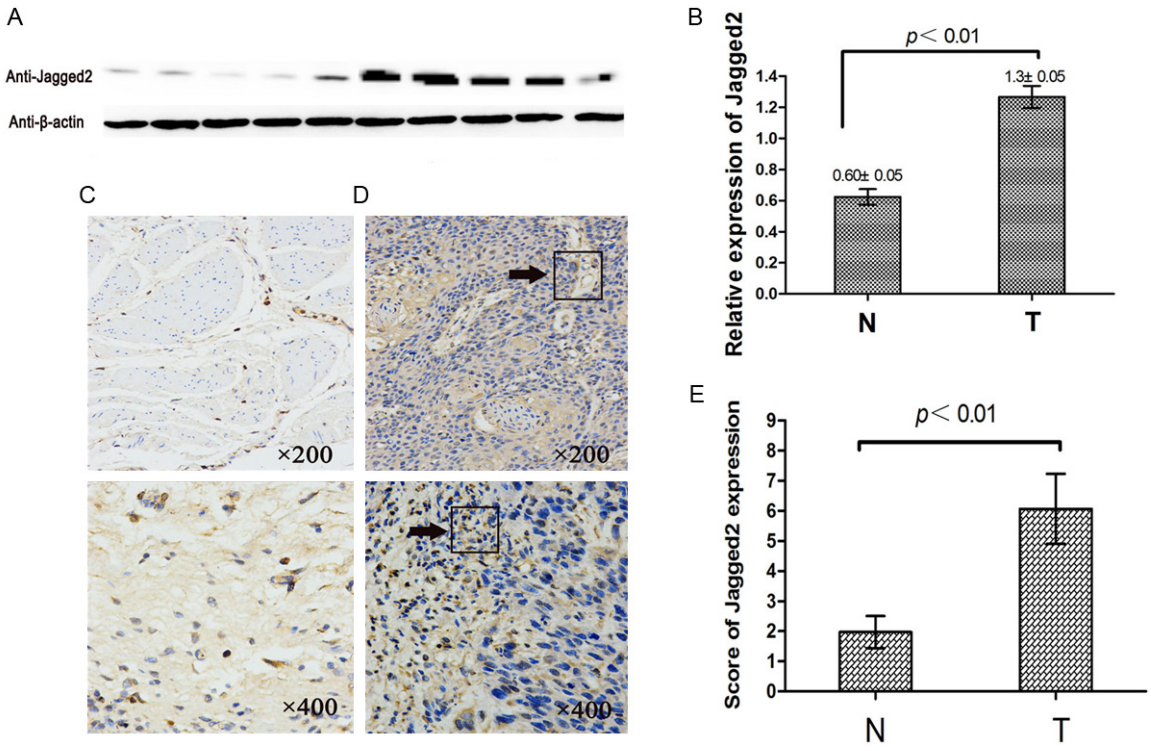

Figure 1. Expression of Jagged2 by Western blotting in 5 bladder cancer tissues and 5 normal bladder tissues. A: Band intensities indicate significant Jagged2 up-regulation in bladder cancer tissues (T) in comparison with the normal bladder tissues (N). β-actin was used as a loading control to assure equal amounts of protein in all lanes. B: The ratio between the optical density of Jagged2 and β-actin of the same tissue was calculated and expressed graphically. Significant differences of Jagged2 expression between tumor and normal tissues were analyzed statistically and Jagged2 expression was obviously greater in bladder cancer tissues (P<0.01). C: Jagged2 immunostaining in the normal bladder tissue. D: Jagged2 immunostaining in the bladder cancer. E: The Jagged2 expression scores in normal bladder tissue and bladder cancer by immunohistochemistry were shown graphically. Significant differences of Jagged2 expression between tumor and normal tissues were analyzed statistically and Jagged2 expression in normal tissues was noticeably greater in bladder cancer tissues (P<0.01).

Table 1. Association of Jagged2 expression with clinico-pathological characteristics of the bladder cancer patients

Parameters	Group	No. of cases (%)	Cases with high Jagged2 expression (%)	χ^2	p
Sex	Male	100 (83.3%)	61 (61.0%)	0.052	0.870
	Female	20 (16.7%)	9 (45.0%)		
Age (years)	<62	42 (35.0%)	18 (42.9%)	2.342	0.147
	≥62	78 (65.0%)	52 (66.7%)		
Histological grade	PUNLMP	7 (5.8%)	0 (0%)		
	Low grade	59 (49.2%)	24 (40.7%)	8.105	0.005
	High grade	54 (45.0%)	46 (85.2%)		
P T	<T1	58 (48.3%)	19 (32.8%)	21.706	<0.001
	≥T1	62 (51.7%)	51 (82.3%)		
Tumor size	<3.5 cm	69 (57.5%)	43 (62.3%)	1.849	0.157
	≥3.5 cm	51 (42.5%)	27 (53.0%)		
Recurrence	Yes	77 (64.2%)	60 (85.7%)	35.182	<0.001
	No	43 (35.8%)	10 (23.3%)		
Metastasis	Yes	37 (30.8%)	31 (83.3%)	13.925	0.001
	No	83 (69.2%)	39 (47.0%)		

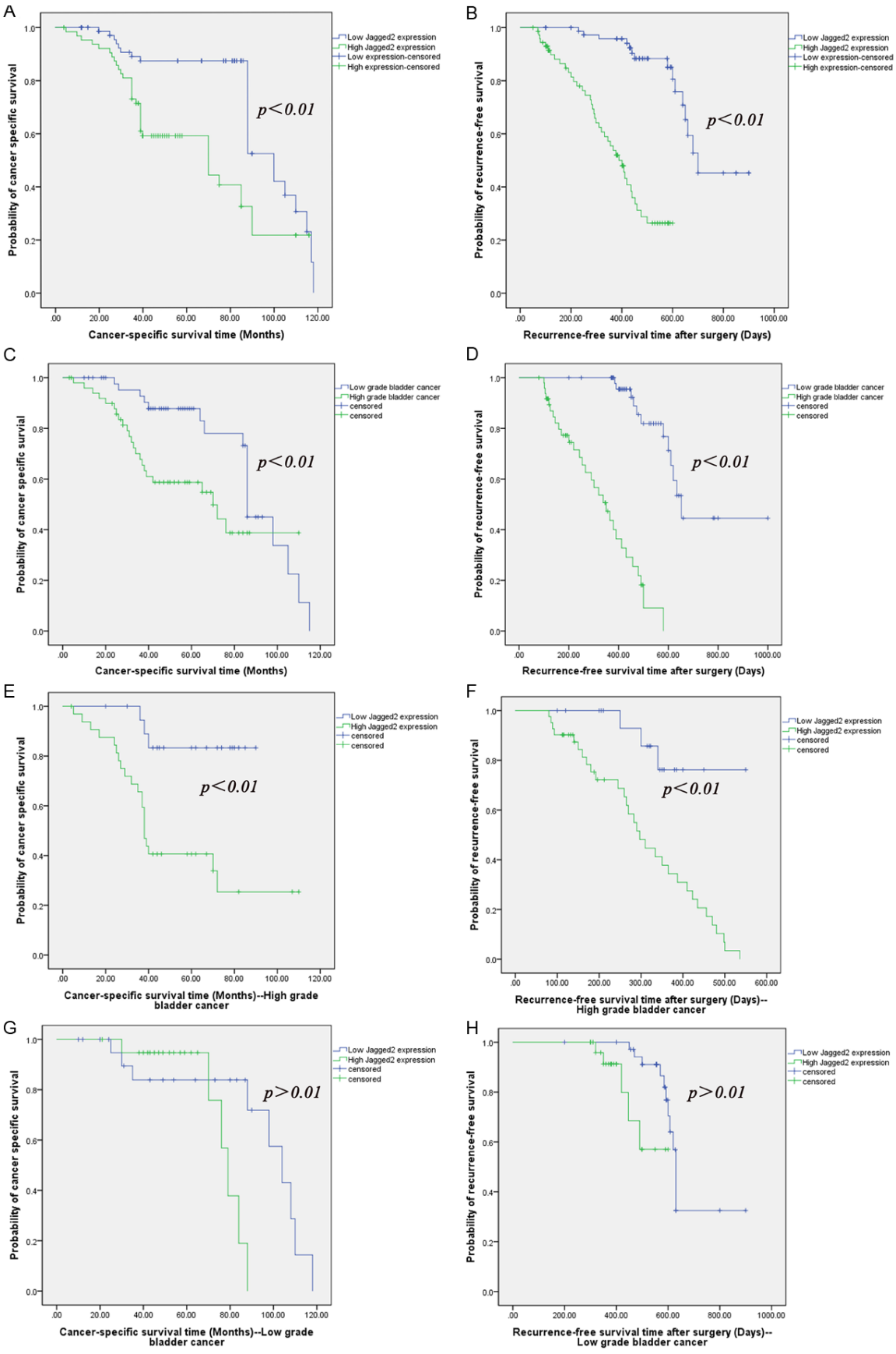
A
Probability of cancer specific survival
Cancer-specific survival time (Months)
Low Jagged2 expression
High Jagged2 expression
Low expression-censored
High expression-censored
p<0.01
B
Probability of recurrence-free survival
Recurrence-free survival time after surgery (Days)
Low Jagged2 expression
High Jagged2 expression
Low expression-censored
High expression-censored
p<0.01
C
Probability of cancer specific survival
Cancer-specific survival time (Months)
Low grade bladder cancer
High grade bladder cancer
censored
censored
p<0.01
D
Probability of recurrence-free survival
Recurrence-free survival time after surgery (Days)
Low grade bladder cancer
High grade bladder cancer
censored
censored
p<0.01
E
Probability of cancer specific survival
Cancer-specific survival time (Months)--High grade bladder cancer
Low Jagged2 expression
High Jagged2 expression
censored
censored
p<0.01
F
Probability of recurrence-free survival
Recurrence-free survival time after surgery (Days)--High grade bladder cancer
Low Jagged2 expression
High Jagged2 expression
censored
censored
p<0.01
G
Probability of cancer specific survival
Cancer-specific survival time (Months)--Low grade bladder cancer
Low Jagged2 expression
High Jagged2 expression
censored
censored
p>0.01
H
Probability of recurrence-free survival
Recurrence-free survival time after surgery (Days)--Low grade bladder cancer
Low Jagged2 expression
High Jagged2 expression
censored
censored
p>0.01

Figure 2. Cancer-specific survival and recurrence-free survival grouped by Jagged2 expression, and calculated by the Kaplan-Meier method. A: Patients with intense Jagged2 expression had significantly lower cancer-specific survival than those with weak Jagged2 expression through log-rank univariate analysis (n=120, P<0.01). B: Patients with intense Jagged2 expression had significantly lower recurrence-free survival than those with weak Jagged2 expression through log-rank univariate analysis (n=120, P=0.028). C: Patients with high grade bladder cancer had significantly lower cancer-specific survival than those with low grade bladder cancer through log-rank univariate analysis (n=120, P<0.01). D: Patients with high grade bladder cancer had significantly lower recurrence-free survival than those with low grade bladder cancer through log-rank univariate analysis (n=120, P<0.01). E: Among patients with high grade bladder cancer, those with intense Jagged2 expression had significantly lower cancer-specific survival than those with weak Jagged2 expression through log-rank univariate analysis (n=54, P=0.001). F: Among patients with high grade bladder cancer, those with intense Jagged2 expression had significantly lower recurrence-free survival than those with weak Jagged2 expression through log-rank univariate analysis (n=54, P<0.01). G: Among patients with low grade bladder cancer, cancer-specific survival with intense Jagged2 expression was not significantly different from that with weak Jagged2 expression through log-rank univariate analysis (n=66, P=0.047). H: Among patients with low grade bladder cancer, recurrence-free survival with intense Jagged2 expression was not significantly different from that with weak Jagged2 expression through log-rank univariate analysis (n=66, P=0.034).

Table 2. The association among Jagged2 expression, *p* T stage, and histological grade with poor prognosis in Cox proportional hazards model

Parameters	Ward	*p*	95% CI
Histological grade	12.476	0.001	1.796-7.832
P T stage	3.472	0.105	0.941-2.319
Jagged2	9.267	0.009	1.324-2.746

or increasing concentrations (1, 2, 3, and 4 μM) of GSIXX (dibenzapine, EMD Millipore, Chicago, IL) were added (no more than 0.5% DMSO final concentration) to cultures for 48 hours. MTT (20 μl, 5 mg/ml) was added to each well and incubated for 4 hours. Then, 200 μl of dimethylsulfoxide was added to each well and the plate was vortexed for 10 minutes at 37°C. Finally, the optical density value (A) of each well was measured at a measurement wavelength of 490 nm using a plate reader (model 680; Bio-Rad, Hertfordshire, UK). Cell growth inhibition ratio was calculated as (1-A_{490} of experimental well/A_{490} of blank control well) ×100%. Each assay was repeated at least 3 times.

Cell cycle assay by flow cytometry

T24 cells in logarithmic growth phase were plated in 25 cm^2 flasks and incubated overnight. Then DMSO vehicle alone (control group) or increasing concentrations (1, 2, 3, and 4 μM) of GSIXX to cultures and they were incubated for 48 hours. Briefly, the attached cells were trypsinized and collected by centrifugation, washed in PBS, and fixed in precold 70% ethanol for 1.5 hours at 4°C. After fixation, the cells were washed in PBS again and centrifuged 5 minutes at 1,000 g. The PBS was discarded and PI was added to a final concentration of 50 μg/ml with dark at 4°C for 30 minutes. Flow-cytometric analysis was performed on the FACS Calibur flow-cytometer (Becton Dickinson, Oxford, UK). Finally, the cell cycle was analyzed using Cell Quest software.

Transwell cell migration assay

Cell migration assay was performed using a 24-well Transwell chamber (Costar, Cambridge, MA). At 48 hours following the treatment of DMSO vehicle alone (control group) or increasing concentrations of GSIXX, T24 cells (1×10^4) were detached and seeded in the upper chamber of a 8 μm pore size insert in the 24-well plate and cultured for another 12 hours. Cells were allowed to migrate forward to DMEM containing 15% FBS in the bottom chamber. The non-migratory cells on the upper membrane surface were removed with a cotton tip, and the migratory cells attached to the lower membrane surface were fixed with 4% paraformaldehyde and stained with hematoxylin. The number of migrated cells was counted in 5 randomly selected high power fields under microscope. Data presented are representative of 3 individual wells.

Statistical analysis

Computerized statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 12.0. Clinical and histopathologic information and the results from the immunohistochemical studies were entered into a database. The significance of Jagged2 expression for tumor recurrence was analyzed by the Kaplan-Meier method, and the differences were evaluated by the log-rank test.

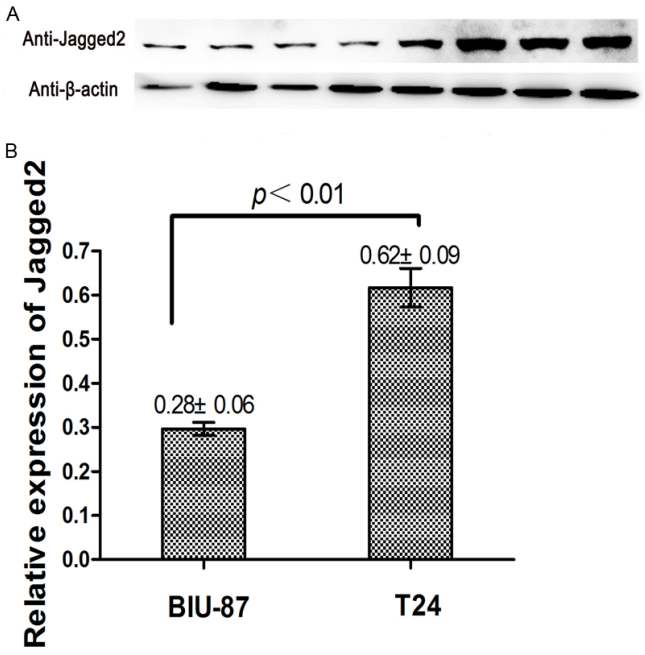

Figure 3. Expression of Jagged2 by Western blotting in bladder cancer BIU-87 cells and bladder cancer T24 cells. A: Band intensities indicate significant Jagged2 up-regulation in bladder cancer T24 cells in comparison with bladder cancer BIU-87 cells. B: The ratio between the optical density of Jagged2 and β-actin of the same sample was calculated and expressed graphically. Significant difference of Jagged2 expression between BIU-87 cells and T24 cells were analyzed statistically and Jagged2 expression was noticeably greater in T24 cells (P<0.01). The data are representative of 3 individual experiments.

The t-test was used to analyze the data from Western blot in the tissues and cells. Groups with the treatment of different concentration's GSIXX were compared by using one way analysis of variance. Multivariable recurrence-free survival analyses were performed with the Cox proportional hazards model. In all statistical analyses, a two-tailed p value <0.05 was considered statistically significant.

Results

Expression of Jagged2 in bladder cancer tissues and normal bladder tissues

Western blot analysis and immunohistochemistry were used to evaluate Jagged2 expression in 60 bladder urothelial carcinoma tissues and 20 normal bladder epithelial tissues. It showed that the increasing Jagged2 expression could be detected in bladder cancer samples in comparison with the normal bladder samples by statistical analysis (p<0.01). The Western blotting of 10 samples are shown in **Figure 1A**, and the optical density of the tumorous (T) and normal (N) tissues is measured and expressed graphically (**Figure 1B**). The immunostaining data about the Jagged2 expressions of normal bladder tissue and bladder cancer are shown in **Figure 1C** and **1D**. The Jagged2 expression level in bladder cancer was noticeably higher than that in normal bladder tissues (p<0.01, **Figure 1E**).

Association between Jagged2 expression and clinicopathologic parameters

To evaluate the significance of Jagged2 high expression in bladder urothelial carcinoma, we investigated the relationship between Jagged2 immunostaining and clinicopathologic features (**Table 1**). Overall, there was no significant relationship between Jagged2 expression and gender (p>0.5), age (p=0.135), and tumor size (p=0.163). However, significant correlations were found between Jagged2 expression and histologic grade (p=0.005), p T stage (p<0.001), recurrence (p<0.001), and metastasis (p<0.005). Therefore, Jagged2 expression status was closely correlated with important histopathologic characteristics (grades and stages) and the recurrence and metastasis of bladder urothelial carcinomas.

High Jagged2 expression was related to recurrence and metastasis of bladder urothelial carcinomas and poor prognosis of the patients

In the follow-up period, 85.7% (60 of 70) of tumors with high Jagged2 expression recurred compared with 36% (18 of 50) of tumors with low Jagged2 expression having recurrence (p<0.001). Furthermore, 44.3% (31 of 70) of tumors with high Jagged2 expression showed metastasis, compared with only 14% (7 of 50) of tumors with low Jagged2 expression having metastasis (p<0.001). Therefore, high expression of Jagged2 was positively associated with the incidence of recurrence and metastasis of bladder urothelial carcinomas.

Kaplan-Meier plots and log-rank tests showed that patients with high Jagged2 expression in their tumor tissues had statistically significant

Table 3. Inhibition rate of different concentrations of GSIXX on the proliferation of T24 cells at different time points

Group	24 h	48 h	72 h
Control	0.0±0.0	0.0±0.0	0.0±0.0
GSIXX 1 μM	8.79±3.28*	15.40±3.92*	19.46±4.88**,*
GSIXX 2 μM	9.34±3.82	18.72±3.79**,*	28.86±4.76**,*
GSIXX 3 μM	18.56±4.37*	35.20±5.74**,*	43.74±5.03*
GSIXX 4 μM	31.72±3.85*	62.40±5.50**,*	70.40±3.28*

*Significant difference ($p<0.01$) compared with former concentration group at the same time point. **Significant difference ($p<0.01$) compared with former time point group with the same concentration.

shorter cancer-specific survival and recurrence-free survival rate compared with those whose tumors had low Jagged2 expression ($p<0.01$; **Figure 2A** and **2B**); patients with high grade bladder cancer had statistically significant shorter cancer-specific survival and recurrence-free survival rate compared with those with low grade bladder cancer ($p<0.01$; **Figure 2C** and **2D**). Moreover, we found that patients with high grade bladder cancer with intense Jagged2 expression had significantly lower cancer-specific survival and recurrence-free survival rates than those with weak Jagged2 expression through log-rank univariate analysis ($p<0.01$; **Figure 2E** and **2F**). However, patients of low grade bladder cancer with intense Jagged2 expression had the same cancer-specific survival and recurrence-free survival rates as those with weak Jagged2 expression through log-rank univariate analysis ($p>0.01$; **Figure 2G** and **2H**).

Log-rank univariate analysis also showed that p T stage ($p>0.01$) and the expression of Jagged2 ($p>0.01$) were significant predictors of the recurrence of bladder tumor. In contrast, there was no significant association between tumor recurrence and other clinicopathologic factors such as gender, age, pathologic grade, and size of tumor. However, when the parameters with significant prognostic impact in univariate analysis were introduced as covariates in Cox proportional hazards model, the expression of Jagged2 and histologic grade had statistically significant independent association with poor prognosis (**Table 2**). Therefore, Jagged2 expression in bladder urothelial carcinomas was inversely associated with poor prognosis of the patients.

Jagged2 expression in bladder cancer cell lines

The above studies have reported that Jagged2 expression was correlated with the metastasis of bladder cancer tissues. Next, Western blotting was used to determine the expression of Jagged2 in human bladder cancer cell lines BIU-87 and T24. The results showed that high invasive bladder cancer T24 cells had intense Jagged2 expression compared with the superficial bladder BIU-87 cells (**Figure 3A** and **3B**). It was further confirmed that Jagged2 expression was positively correlated with the tumor malignancy grade.

GSIXX, Jagged2 inhibitor, inhibited the proliferation and invasion of T24

For further research on the Jagged2's role in bladder cancer, the inhibitor of Jagged2, GSIXX, was applied to treat bladder cancer T24 cells. With the increasing concentration of GSIXX, we found that the cell proliferative and invasive activities both decreased significantly (**Table 3**, **Figure 4A** and **4B**). Moreover, the data of FCS showed that not only the cell growth rate was decreased, but also the cell cycle was blocked at G_2/M stage (**Table 4**, **Figure 4C**). It demonstrated that Jagged2 played an important function on the bladder cancer cells' proliferation by regulating the cancer cell cycle from G_1/S to G_2/M and probably promoted the invasion and metastasis of bladder cancer.

Discussion

Notch signaling is frequently observed in solid tumors and its critical role in tumor pathology and progression has been issued [19]. However, less is known about the expression status of Notch ligand in tumor and tumor-associated prognosis. Results from microarray data in breast cancer patients indicated that Jagged2 was the only ligand, and its expression was significantly correlated with metastasis-free survival [13]. Latest studies have demonstrated that over-expressions of Jagged2 in several human cancers are correlated with tumor metastasis and patient survival. It has been reported that the level of Jagged2 in tumor samples could be linked to the prognosis of

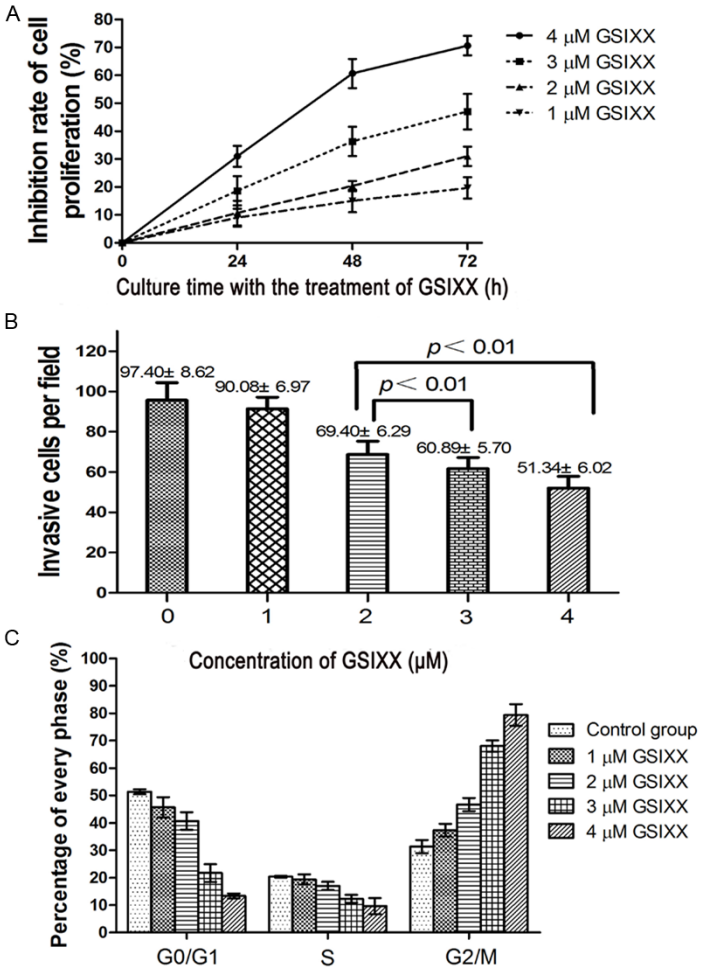

Figure 4. Proliferation and invasion ability of T24 were inhibited by GSIXX. A: MTT assay was used to examine T24 cell proliferation. Cell proliferation was inhibited on dose-dependent correlation with the increasing concentration of GSIXX. B: Transwell invasion assay was used to examine T24 cell migration. Cell invasion was inhibited on dose-dependent correlation with the increasing concentration of GSIXX. C: FCS was used to examine T24 cell cycle. With the increasing concentration of GSIXX, the number of T24 cells at G_0/G_1 stage decreased and increased at G_2/M stage, but the number of T24 at S stage did not noticeably change. The data are representative of 3 individual experiments.

Table 4. Flow cytometry analysis of T24 cells treated with different concentrations of GSIXX

Group	G_0/G_1	S	G_2/M
Control	50.2%±2.1%	19.3%±1.3%	31.8%±2.7%
GSIXX 1 μM	46.8%±1.1%	18.6%±2.8%	36.6%±1.2%
GSIXX 2 μM	39.5%±2.7%*	15.2%±1.7%	46.2%±1.5%*
GSIXX 3 μM	22.1%±1.5%*	12.7%±1.6%*	65.9%±0.7%*
GSIXX 4 μM	12.8%±1.3%*	10.4%±0.9%*	78.5%±2.4%*

*p<0.05 vs. control.

patients with breast cancer [20]. Our data also show that high expression level of Jagged2 is strongly correlated with clinicopathologic parameters in bladder carcinoma patients. Several follow-up studies showed that quantification of Jagged2 expression levels had a prognostic value for a variety of cancers, including breast cancer, and melanoma [21, 22]. Jagged2 expression has been reported as a valuable prognostic marker and may serve as a novel therapeutic target for metastatic breast cancer [13]. We also find that high Jagged2 expression is related to recurrence and metastasis of bladder urothelial carcinomas and poor prognosis of the patients. The correlation between Jagged2 expression with clinical stage and histologic grade of a tumor has the potential to aid clinicians in their search for improving treatment decisions for different cancer patients. Novel evidence demonstrated that Jagged2 could promote lung cancer metastasis through increasing GATA binding factors and suppressing the expression of microRNA-200 [14]. These findings strengthened the link between Jagged2 and tumor metastasis. Jagged2 is considered as a "switcher" for epithelail-to-mesenchmal transition, and EMT was regarded as a vital factor in tumor metastasis [23]. Several strategies for inhibition of Jagged2 have been tested in cancer therapeutic trials [24, 25]. For example, phenylhexyl isothiocyanate was used as inhibitor of Jagged2 in multiple myeloma cell RPMI8226 in vitro [26]. Inhibition of Notch signaling through RNAi targeting JAG2 or the gamma-secretase Notch inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester (DAPT) preferentially inhibited the neoplastic state in clitured human P493-6 B cells, which have a pre-neoplastic state dependent on the Epstein-Barr viral EBNA2 protein and a neoplastic state with ectopic inducible Myc [27]. In another

model, Striking overexpression of Jagged2 was detectable in the vast majority of pancreatic cancer cell lines; while transient pretreatment of pancreatic cancer cells with GSI-18 resulted in depletion in the proportion of tumor-initiating aldehyde dehydrogenase-expressing subpopulation and was associated with inhibition of colony formation in vitro and xenograft engraftment in vivo, underscoring a requirement for the Notch-dependent aldehyde dehydrogenase-expressing cells in pancreatic cancer initiation [12]. These data suggest that inhibition of Jagged2 activity is crucial for the observed growth inhibition and apoptosis induction. The effect of GSIXX, a small molecule inhibitor of Jagged2, was examined in our experiment, with the aim of observing the consequences of Jagged2 inhibition in bladder cancer cells. Incubation of bladder cancer T24 cells with increasing concentrations of GSIXX was found to decrease cell proliferation and invasion in a dose-dependent manner, and induce the cell G_2/M stage arrest.

In conclusion, we propose that Jagged2 expression status is closely correlated with important histopathologic characteristics (grades and stages), recurrence, and metastasis of bladder urothelial carcinomas. Jagged2 can be a potential target for the treatment of bladder carcinoma. Furthermore, Jagged2 plays an important function on the bladder cancer cells' proliferation by regulating the cancer cell cycle from G_1/S to G_2/M and probably promoting the invasion and metastasis of bladder cancer. Further efforts to investigate the mechanism of Jagged2 involved in bladder cancer metastasis are warranted.

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Disclosure of conflict of interest

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

Abbreviations

PUNLMP, papillary urothelial neoplasms of low malignant potential; PBS, phosphatebuffered saline; ELISA, enzyme-linked immuno sorbent assay; DMSO, dimethylsulfoxide; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester; IHC, Immunohistochemistry; RNAi, RNA interfere; EMT, epithelial-mesenchymal transition; DMEM, Dulbecco's modified eagle medium; MTT, Methyl Thiazolyl Tetrazolium; MDA-MB-435, a type of frequently-used breast cancer cell line.

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