

Keywords: prognostic factor; Bcl-xL; upper urinary tract; urothelial carcinoma; bafilomycin; animal model

Prognostic significance of Bcl-xL expression and efficacy of Bcl-xL targeting therapy in urothelial carcinoma

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Background: Bcl-xL has an important role in the control of cell death through its inhibition of apoptosis. The aim of this study was to investigate the clinicopathological significance of Bcl-xL in upper urinary tract urothelial carcinoma (UTUC) and the therapeutic effect of targeting Bcl-xL protein in urothelial carcinoma (UC) cells.

Methods: We evaluated the immunohistochemical expression of Bcl-xL in 175 UTUC patients to determine the clinical role of Bcl-xL expression in clinical outcome. We used bafilomycin A1 (BMA) as a specific inhibitor of Bcl-xL to examine the biological effects in UC cells *in vitro* and *in vivo*.

Results: Immunohistochemical analysis of Bcl-xL expression revealed that patients with a high Bcl-xL score had a significantly lower 5-year cancer-specific survival (CSS) rate (53.2%) than those with a low Bcl-xL score (77.2%) ($P=0.0011$). Multivariate analysis indicated that a high Bcl-xL score was an independent prognostic factor of CSS ($P=0.023$). BMA inhibited UMUC-3 cell proliferation *in vitro* by induction of apoptosis. Treatment with BMA significantly inhibited tumour growth in UMUC-3 tumours in this mouse xenograft model accompanied by an elevated apoptosis induction.

Conclusion: Bcl-xL appears to be a significant molecular marker for the prognosis of UTUCs. Targeting Bcl-xL may be a promising therapeutic strategy for patients with UC.

Bladder cancer is the most common neoplasm occurring along the urothelium. By contrast, upper urinary tract urothelial carcinomas (UTUCs) are relatively rare, accounting for approximately 10% of all renal tumours and approximately 5% of all urothelial carcinomas (UCs; Oosterlinck, 2007). Radical nephroureterectomy (RNU) with bladder cuff excision is the standard procedure for UTUC. As UTUCs are a relatively rare type of tumour, there are few molecular markers known to predict the disease prognosis in UTUC patients (Hall *et al*, 1998; Nakanishi *et al*, 1998; Fromont *et al*, 2005; Miyata *et al*, 2005; Kashibuchi *et al*, 2006; Catto *et al*, 2007; Jeong *et al*, 2009; Kosaka *et al*, 2010).

Although 20–30% of bladder cancers have the invasive stage at the initial presentation, approximately 60% of UTUC are diagnosed at the invasive stage (Hall *et al*, 1998; Kirkali

et al, 2005). Even if RNU was performed for UTUC patients, >20% of UTUC patients experienced extravesical disease recurrence or distant metastasis during follow-up (Rink *et al*, 2012). Therefore, it is important to predict which UTUC patients are most likely to have recurrence and poor survival and to conduct follow-up in order to classify their risks.

Bcl-xL is one of the Bcl-2 family proteins and has an important role in controlling cell death and in the response of cancer cells to chemotherapeutic drugs or radiation therapy (Cotter, 2009). Bcl-xL molecules may inhibit apoptosis by maintaining the permeabilisation status or stabilisation of the outer mitochondrial membrane (Boise *et al*, 1993). Targeting the anti-apoptotic Bcl-2 family proteins can induce apoptosis and thus overcome drug resistance to cancer chemotherapy (Kang and Reynolds, 2009). Although

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Bcl-2 was not overexpressed frequently in UC of urinary bladder (Kirsh *et al*, 1998), Bcl-xL is expressed at a high frequency (Lebedeva *et al*, 2001; Korkolopoulou *et al*, 2002), therefore, it might become the target of treatment in UC. Several studies have evaluated the prognostic role of Bcl-xL protein expression in bladder carcinoma, especially for superficial bladder carcinoma (Gabriel *et al*, 2008; Bolenz *et al*, 2009). However, the status of Bcl-xL expression in UTUC tissues and its prognostic significance are unclear.

Vacuolar H⁺-ATPase (V-ATPase) is the specific proton pump of cells and its inhibitors have been shown to induce apoptosis of cancer cell lines (Ohta *et al*, 1998; Nakashima *et al*, 2003; Morimura *et al*, 2008). More recently, Bcl-xL protein has been shown to be the target for V-ATPase inhibitors such as bafilomycin A1 (BMA) in cancer cells (Sasazawa *et al*, 2009). Therefore, BMA may regulate the function of Bcl-xL protein in cancer cells and exhibit an anticancer effect. Whether targeting therapy for Bcl-xL proteins, such as BMA, could induce apoptosis and has an anti-tumour effect in UC cells have never been evaluated.

Therefore, in the present study, we evaluated (i) the association between the tumour characteristics of 175 cases of UTUC and the expression of Bcl-xL by immunohistochemistry with slides re-reviewed by genitourinary pathologists to determine the clinical role of Bcl-xL expression in survival in UTUC patients, and (ii) examined whether targeting therapy for Bcl-xL using BMA could induce apoptosis and have a therapeutic effect on UC cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Immunohistochemical evaluation of Bcl-xL in UTUC human samples. A total of 201 patients had undergone surgical treatment for UTUC at Keio University Hospital from 1999 to 2007. This study design was approved by the ethics review board of Keio University and the medical records of patients were retrospectively reviewed. The median follow-up period was 35.0 months and mean patient age was 66.2 years old (range, 36–89). RNU was performed in 196 patients and partial ureterectomy was performed in 5 patients. Local lymph node dissection was performed in patients with a suspected adenopathy observed pre- or intraoperatively. Extended lymph node dissection was not routinely performed. We identified a total of 175 patients with pathological Ta-T4N0M0 tumours (pTa-4N0M0) in our study population (pTa-pT1: 57 patients, pT2: 37 patients, pT3: 77 patients, pT4: 4 patients). Patients with distant metastasis at diagnosis or positive lymph node involvement were excluded from the analysis. Cisplatin-based adjuvant chemotherapy regimens were administered to 26 patients (14.9%). The patients were followed postoperatively with urinary cytology every 3 months for 2 years and every 6 months thereafter. Computed tomography or magnetic resonance imaging as well as cystoscopy were performed every 6 months for 5 years and annually thereafter.

All tumour specimens were fixed in 10% formalin and embedded in paraffin, and all slides were re-reviewed by uropathologists. Tumours were staged according to the American Joint Committee on Cancer–Union Internationale Contre le Cancer TNM classification. Tumour grading was assessed according to the 1998 WHO/International Society of Urologic Pathology consensus classification (Epstein *et al*, 1998). Lymphovascular invasion (LVI) was defined as the presence of tumour cells within an endothelium-lined space without underlying muscular walls.

Sections (4 µm) of formalin-fixed and paraffin-embedded material were analysed. The sections were deparaffinised in xylene and rehydrated in graded alcohols and distilled water. After antigen retrieval with Target Retrieval Solution (pH 9.0)

(Dako, Glostrup, Denmark), endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 30 min followed by washing with distilled water. To bind nonspecific antigens, the sections were incubated with 5% skim milk for 15 min. The sections were incubated with a primary Bcl-xL mouse monoclonal antibody (1:100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at room temperature for 1 h. After washing with PBS, they were incubated with secondary antibodies for 30 min. The antibody was detected using the avidin–biotin complex peroxidase method. Color was developed with 3,3'-diaminobenzamine tetrahydrochloride in 50 mmol l⁻¹ Tris-HCl (pH 7.5) containing 0.005% hydrogen peroxide. The specificity of the antibody was examined using blocking peptides (Santa Cruz Biotechnology) to inhibit adsorption for Bcl-xL antibody, resulting in a significantly reduced staining reaction as compared with the staining with the primary antibody (Figures 1E and F). The sections were counterstained with hematoxylin.

The results of Bcl-xL immunostaining were scored as described previously (Krajewska *et al*, 1996). To evaluate Bcl-xL staining, cancer cells with positive staining in the cytoplasm were counted in at least 10 representative fields selected randomly by a uro-pathologist who was unaware of the clinicopathological data and clinical outcomes of the patients. The percentage of positive tumour cells was graded as follows: 0, none; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%. The intensity of the tumour cells was rated as follows: 0, none; 1, weak; 2, moderate; and 3, intense. The score was calculated by multiplication of the grading score of the percentage of positive tumour cells and that of intensity, and is expressed in the range of 0–12. To evaluate the Bcl-xL expression and the prognostic value of UTUC patients, we divided the Bcl-xL score into two groups according to the intensity of Bcl-xL expression. A high Bcl-xL score and a low Bcl-xL score was defined as 9–12 and 0–8, respectively.

Cell culture and materials. The UMUC-3 UC cell line was routinely maintained in RPMI-1640 (Invitrogen, Tokyo, Japan) with 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO₂ atmosphere. UMUC-3 was obtained from the American Type Culture Collection. BMA was purchased from Wako Pure Chemical Industries (Osaka, Japan). BMA was dissolved in dimethyl sulphoxide (DMSO) and diluted to appropriate concentrations with culture medium.

Cell growth assay. UMUC-3 cells were plated in 96-well plates, allowed to adhere for 24 h, and then treated with various concentrations of BMA. Cells treated with the same concentrations of DMSO served as controls. At the end of the incubation period, cytotoxicity was determined using WST-1; 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulphonate (Takara Bio Inc., Shiga, Japan). The absorbance value of each well was determined at 450 nm with a 650 nm reference beam by a microplate reader (Bio-Rad Laboratories, Inc., Tokyo, Japan). The BMA dose used in the cell viability assay was the same as that of previous reports in gastric cancer and pancreatic cancer (Ohta *et al*, 1998; Nakashima *et al*, 2003). These experiments were repeated on different days when new cells had grown.

Western blot analysis. Whole-cell extracts were obtained using radioimmunoprecipitation assay buffer (50 mmol l⁻¹ Tris-HCl (pH 7.5), 150 mmol l⁻¹ NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) containing protease inhibitors. For western blot analysis, 50 µg of total protein for Bcl-xL, cytochrome c, and caspase-3 (Santa Cruz Biotechnology) from each sample were loaded on 12.5% SDS–polyacrylamide gels. Immunoblotting was also performed according to a standard method. Proteins were transferred onto a polyvinylidene difluoride membrane in blocking solution (5% nonfat dry milk in TBS containing 0.1% Tween 20). Primary antibodies for Bcl-xL (1:200 dilution), cytochrome c (1:200

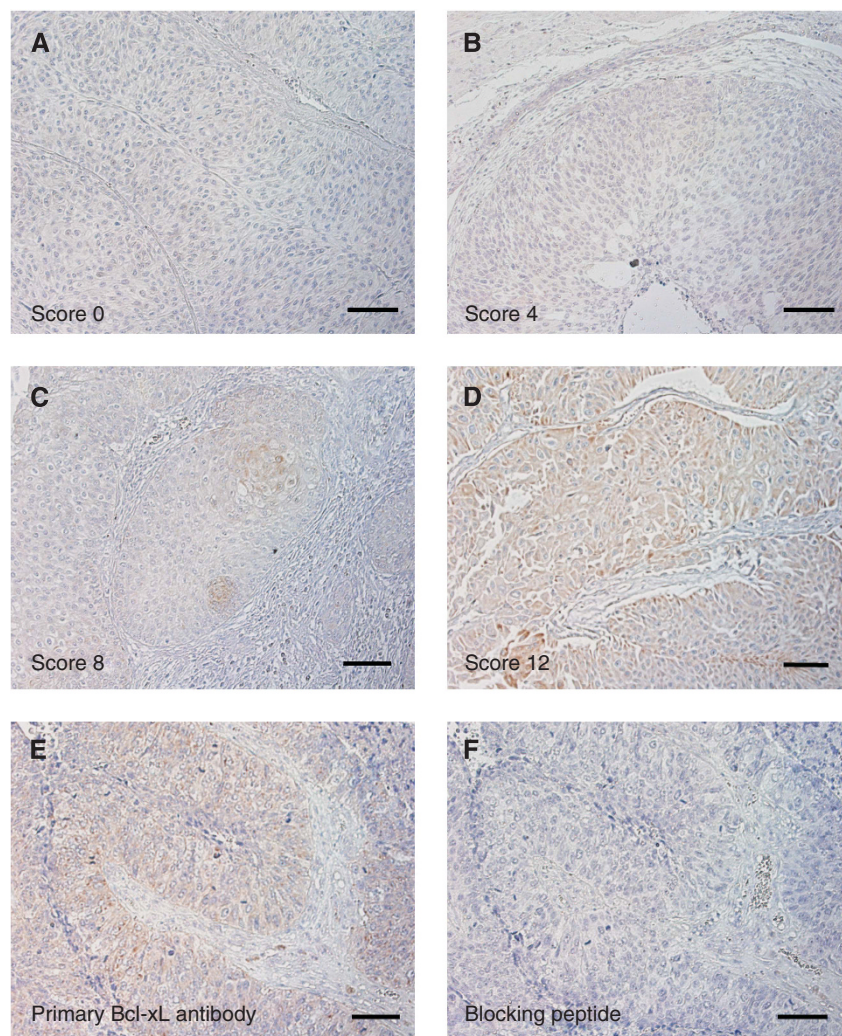


Figure 1. Immunostaining of Bcl-xL protein expression in upper urinary tract carcinoma tumour tissue. (A) Bcl-xL score 0 points (negative staining), (B) Bcl-xL score 4 points, (C) Bcl-xL score 8 points, and (D) Bcl-xL score 12 points. (E) Immunostaining with primary antibody for Bcl-xL, (F) addition of blocking peptide for Bcl-xL to primary antibody for Bcl-xL, resulting in a significantly reduced staining reaction (bars = 100 μm).

dilution), and caspase-3 (1:200 dilution) were used for the study. After washing, the membranes were incubated for 1.5 h at room temperature linked with peroxidase secondary antibody (Dako, Glostrup, Denmark), and then the proteins were visualised on X-ray film using an electrochemiluminescence western blotting detection kit (PerkinElmer Life Science, Waltham, MA, USA).

Flow cytometric analysis for the detection of apoptosis. Flow cytometric analysis was performed using TUNEL assay for detecting apoptosis and BrdU assay for cell cycle analysis. Briefly, cells (1×10^6) were plated in 100 mm dishes and allowed to attach overnight. They were then treated with 5 nM of BMA for 12 h. Next, the cells were harvested and fixed in 70% ethanol at 4 °C overnight, resuspended in PBS containing 0.05 mg ml⁻¹ RNase A (Sigma Chemical, St. Louis, MO, USA), and incubated at room temperature for 30 min. After washing, the cells were stained with FITC-labeled BrdU (BD Biosciences, Franklin Lakes, NJ, USA) and propidium iodide and analysed by flow cytometry (Beckman Coulter, Fullerton, CA, USA). TUNEL assay was performed using ApopTag Kits (Sigma Chemical) according to the manufacturer's protocol, and apoptosis was detected by flow cytometry (Beckman Coulter).

Small interfering RNA (siRNA). Bcl-xL expression was transiently downregulated using the following predesigned duplex siRNA directed against Bcl-xL (siBcl-xL; Ambion, Carlsbad, CA, USA). The sense sequences of siRNA for Bcl-xL were as follows: siBcl-xLA, 5'-AUACUUUUGUGGAACUCUAtt-3'; and siBcl-xLB, 5'-GGAACUCUAUGGGAACAAUtt-3'. UMUC-3 cells were cultured in antibiotic-free medium overnight at 37 °C in 5% CO₂ and then cells were transiently transfected with 20 nmol of siBcl-xLA and siBcl-xLB using Lipofectamine 2000 (Invitrogen Co., Tokyo, Japan). After 4 h, siRNA was removed by replacing the culture medium with fresh RPMI 1640 containing 10% FBS, and cells were cultured for additional 48–72 h. A mock-transfection control was prepared using the transfection reagent only.

Treatment in vivo. BALB/c mice, 6 weeks of age with an average body weight of 20 g, were purchased from Sankyo Laboratory Service (Tokyo, Japan). Mice were housed under specific pathogen-free conditions. All of the procedures involving animals and their care in this study were approved by the Animal Care Committee of Keio University in accordance with institutional and the Japanese government guidelines for animal experiments. All mice were inoculated subcutaneously (s.c.) in the flank with 100 μl (1×10^6 cells) of UMUC-3 cells. When the mice had developed palpable

tumours, they were randomly assigned to two groups, each consisting of eight animals. An animal was then given an intraperitoneal injection of 1 mg kg^{-1} of BMA or vehicle control every 4 days for 24 consecutive days. The animals were carefully monitored, and the tumour was measured every 4 days. The mice were killed, and the tumours were collected 28 days after treatment. The tumour volume (V) was calculated according to the formula $V = AB^2/2$, where A is the greatest diameter and B is the diameter at the point perpendicular to A .

For histological examinations, tumour specimens were fixed in 4% paraformaldehyde, processed by routine methods, embedded in paraffin wax, and sectioned at $4 \mu\text{m}$. Immunohistochemical staining with TUNEL assay was carried out on sections of formalin-fixed paraffin wax-embedded tissue. Apoptosis was measured using a commercially available *in situ* apoptosis detection kit (Takara Bio Inc., Shiga, Japan). Visualisation of the immunoreaction was performed with 0.06% 3, 3'-diaminobenzidine (DAB; Sigma Chemical). A dark accumulation of DAB in the nuclei indicated a positive reaction for TUNEL.

Statistical analysis. The differences between the Bcl-xL score and clinicopathological variables were analysed using the Mann-Whitney U test. Cancer-specific survival (CSS) calculated by the Kaplan-Meier method was evaluated using the log-rank test. We used Cox's proportional hazards regression analysis to assess the prognostic indicators that included age, gender, tumour stage, grade, tumour location, LVI, and Bcl-xL score for CSS and bladder recurrence-free survival. The difference between the two groups in *in vitro* study and in the animal model was assessed with the Mann-Whitney U test. The level of statistical significance was set at $P < 0.05$. These analyses were performed with the SPSS Version 16.0 statistical software package (SPSS Corporation, Chicago, IL, USA).

RESULTS

Clinical role of Bcl-xL in UTUC human samples

Bcl-xL expression in UTUC. Immunohistochemical evaluation was performed to detect the level of Bcl-xL protein expression in UTUC tissue samples from 175 cases. Representative images for Bcl-xL scores of 0, 4, 8, and 12 are shown in Figures 1A, B, C, and D, respectively. Positive staining of Bcl-xL protein was mainly located in the cytoplasm of tumour cells in UTUC tissues. Overall, 164 patients (93.7%) had positive immunostaining of Bcl-xL protein and 11 tumour tissues showed negative staining (score 0). The mean \pm s.d. of the Bcl-xL score was 6.50 ± 3.68 in all human samples. The Bcl-xL score was 0–4 in 70 tumour tissues, 5–8 in 56, and 9–12 in 49. The Bcl-xL score for high tumour stages \geq pT2 was 7.36 ± 3.54 , which was significantly higher than that for low tumour stages $<$ pT2 (4.70 ± 3.31 , $P < 0.001$) (Table 1). Patients with a high tumour grade had a higher Bcl-xL score (7.04 ± 3.74) compared with patients with a low tumour grade (5.56 ± 3.40 , $P = 0.007$). Positive LVI was more associated with a higher Bcl-xL score (7.89 ± 3.43) than negative LVI (5.84 ± 3.62 , $P = 0.001$). However, there were no significant differences in age (< 65 or ≥ 65 years: $P = 0.085$) or tumour location (pelvis or ureter: $P = 0.993$) with respect to the Bcl-xL score.

Prognostic significance of Bcl-xL score in CSS of UTUC patients. Kaplan-Meier curve analysis demonstrated that patients with a high Bcl-xL score (Bcl-xL score ≥ 9) had a significantly lower CSS than those in the low Bcl-xL score group (Bcl-xL score < 9) ($P = 0.0011$) (Figure 2A). The 5-year CSS in the high Bcl-xL score group and low Bcl-xL score group was 53.2% and 77.2%, respectively.

Table 1. Association between clinicopathological characteristics and level of Bcl-xL protein expression in 175 UTUC patients

Variables	n	Bcl-xL score	P
Age			
<65 years	73	6.88 ± 3.68	0.085
≥ 65 years	102	5.96 ± 3.63	
Gender			
Male	131	6.17 ± 3.59	0.031
Female	44	7.48 ± 3.80	
Tumour stage			
<pT2	57	4.70 ± 3.31	<0.001
\geq pT2	118	7.36 ± 3.54	
Grade			
Low	64	5.56 ± 3.40	0.007
High	111	7.04 ± 3.74	
Tumour location			
Pelvis	103	6.48 ± 3.59	0.993
Ureter	72	6.53 ± 3.83	
LVI			
Negative	119	5.84 ± 3.62	0.001
Positive	56	7.89 ± 3.43	

Abbreviations: LVI = lymphovascular invasion; UTUC = urinary tract urothelial carcinoma.

In a subgroup of patients with pT2 or greater UTUC tumour ($n = 118$, Figure 2C), there were significant differences in CSS between the high and low Bcl-xL score groups ($P = 0.0227$). The 5-year CSS in \geq pT2 patients in the high Bcl-xL score group and low Bcl-xL score group was 48.7% and 67.4%, respectively. In a subgroup of patients with a tumour $<$ pT2 ($n = 57$, Figure 2D) and low-grade tumour patients ($n = 64$, Figure 2F), there were no significant differences in CSS between the two Bcl-xL groups. In a subgroup of patients with a high-grade UTUC tumour ($n = 111$, Figure 2E), there were significant differences in CSS between the high and low Bcl-xL score groups ($P = 0.0327$). The 5-year CSS in high-grade patients with a high Bcl-xL score and a low Bcl-xL score was 45.0% and 64.9%, respectively. Furthermore, in a subgroup of patients who were LVI positive ($n = 56$), a significant difference in CSS between the high and low Bcl-xL score groups was observed ($P = 0.0074$; Figure 2G). The 5-year CSS in LVI-positive patients with a high Bcl-xL score and a low Bcl-xL score was 20.1% and 51.5%, respectively. By contrast, no significant difference in CSS was observed between the two Bcl-xL groups in patients who were LVI negative ($n = 119$, Figure 2H).

Bladder recurrence was observed in 72 patients (41.1%), and the median bladder recurrence-free period was 9.5 months during follow-up after RNU. Kaplan-Meier curve analysis demonstrated that there was no significant difference between the high and low Bcl-xL score groups with respect to the bladder recurrence-free rate ($P = 0.3276$) (Figure 2B). Furthermore, in a subgroup analysis according to tumour grade, pT stage, or LVI status, no differences in the bladder recurrence rate between the high and low Bcl-xL score groups were observed (data not shown).

Table 2 shows the results of univariate and multivariate Cox regression analyses with regard to the CSS and bladder recurrence-free survival. Univariate analysis revealed that tumour stage, tumour grade, presence of LVI, and Bcl-xL score were significantly

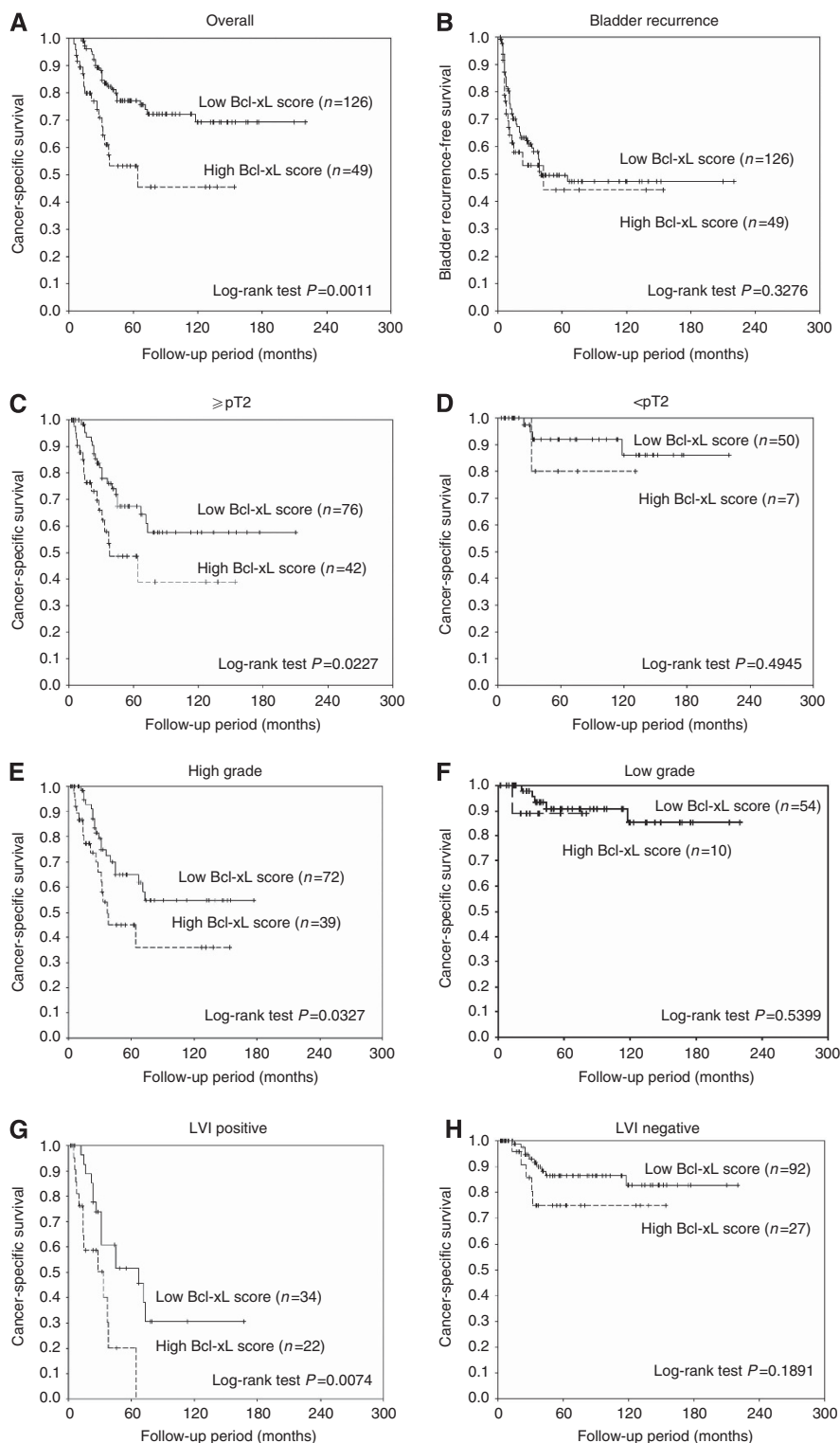


Figure 2. Kaplan–Meier curves of cancer-specific survival (CSS) or bladder recurrence according to the expression level of Bcl-xL score. (A) CSS curve in overall patients, (B) bladder recurrence-free survival curve in overall patients, (C) CSS in patients with \geq pT2 tumour stage, (D) CSS in patients with $<$ pT2 tumour stage, (E) CSS in high-grade tumour patients, (F) CSS in low-grade tumour patients, (G) CSS in lymphovascular invasion (LVI)-positive patients, and (H) CSS in LVI-negative patients.

associated with CSS. Multivariate analysis revealed that the presence of LVI and a high Bcl-xL score were independent prognostic factors of CSS ($P < 0.001$ and $P = 0.023$, respectively). No clinicopathological feature or Bcl-xL score was independently associated with bladder recurrence-free survival.

Therapeutic efficacy of targeting therapy for Bcl-xL using BMA in *in vitro* study

Cell viability assay of UMUC-3 cells treated by BMA. On the basis of the prognostic value of Bcl-xL expression in UTUC

Table 2. Univariate and multivariate analysis for CSS and bladder recurrence-free survival in 175 UTUC patients

	Cancer-specific survival			Bladder recurrence-free survival		
	Univariate analysis	Multivariate analysis		Univariate analysis	Multivariate analysis	
	P	HR (95% CI)	P	P	HR (95% CI)	P
Characteristics						
Age (≥ 65 / < 65) years	0.250		NS	0.969		NS
Gender (male/female)	0.376		NS	0.291		NS
Tumour stage ($\geq pT2$ / $< pT2$)	0.001		NS	0.458		NS
Grade (high/low)	< 0.001		NS	0.977		NS
Tumour location (pelvis/ureter)	0.231		NS	0.169		NS
LVI (positive/negative)	< 0.001	4.660 (2.348–9.247)	< 0.001	0.746		NS
Bcl-xL score (high/low)	0.002	2.075 (1.107–3.892)	0.023	0.337		NS

Abbreviations: CI = confidence interval; CSS = cancer-specific survival; HR = hazard ratio; LVI = lymphovascular invasion; NS = not significant; UTUC = urinary tract urothelial carcinoma.

patients, we investigated whether targeting therapy for Bcl-xL would have a therapeutic effect on UC cells by using BMA, which specifically inhibits Bcl-xL expression. Almost all UC cell lines tested expressed Bcl-xL protein. In those cell lines, UMUC-3 cells showed one of the highest expression levels of Bcl-xL (Figure 3A). Therefore we decided to use UMUC-3 cells for this study.

UMUC-3 cells were grown in the absence or presence of various concentrations of BMA for 48 and 72 h (Figures 3B and C). In UMUC-3 cells, the mean cell viability following treatment with 5 and 10 nM BMA for 48 h was $60.0 \pm 4.5\%$ ($P < 0.01$) and $47.5 \pm 4.8\%$ ($P < 0.01$), respectively, compared with vehicle control. Similar results were observed 72 h after BMA exposure; the mean cell viability following treatment with 5 and 10 nM BMA was $41.2 \pm 4.1\%$ ($P < 0.01$) and $29.9 \pm 3.8\%$ ($P < 0.01$), respectively, compared with vehicle control.

Apoptosis and cell-cycle assay. The TUNEL and cell-cycle assays were performed to detect apoptotic cells and cell-cycle distribution after 5 nM BMA treatment. Higher levels of apoptotic cells were observed after 5 nM BMA treatment (36.4% of apoptotic cells in BMA treatment, Figure 3E vs 0.17% of those in vehicle control, Figure 3D). In the BrdU assay, cancer cells accounted for 57.6% of cells (Figure 3G) in the sub-G1 phase of the cell cycle compared with 4.4% for control cells (Figure 3F).

Effect of BMA on Bcl-xL and apoptotic-related protein expression. Western blot analysis was performed to confirm whether BMA had an effect on Bcl-xL expression and the related apoptotic protein (Figure 3H). Bcl-xL protein was expressed in untreated control UMUC-3 cells. A dose-dependent decrease in Bcl-xL protein expression was observed after 5 and 10 nM BMA treatment. Caspase-3 expression and cytochrome *c* protein expression were increased in the BMA treatment group compared with the controls.

Effect of siRNA for Bcl-xL in UMUC-3 cells. UMUC-3 cells were transiently transfected with 20 nM of siBcl-xLA and siBcl-xLB. To evaluate the inhibition of Bcl-xL protein, we performed western blot analysis for cells treated with these siRNAs. siBcl-xLA and siBcl-xLB demonstrated significant reduction of Bcl-xL expression compared with control. Figure 3I shows the results of western blotting for Bcl-xL protein, 48 h after transfection with 20 nM siBcl-xLA and 20 nM siBcl-xLB. Cell viability assay using WST-1 reagent revealed significant inhibition of cell viability in UMUC-3 cells 72 h after being treated with 20 nM of these siRNAs. The inhibition

of cell viability of UMUC-3 cells transfected with siBcl-xLA and siBcl-xLB was $80.8 \pm 3.0\%$ ($P < 0.05$) and $81.9 \pm 3.7\%$ ($P < 0.05$), respectively, compared with control after 72 h (Figure 3J).

Anti-tumour effect of BMA in UC xenograft model. Based on the *in vitro* findings, we tested the anti-tumour effects of BMA in an *in vivo* study using a UMUC-3 subcutaneous tumour model. The results of this *in vivo* study demonstrated a significant decrease in tumour growth in BMA-treated mice compared with control mice on days 16, 20, and 24 after the start of BMA treatment (Figure 4A). The mean \pm s.d. of tumour volume was $1300 \pm 260 \text{ mm}^3$ in the BMA-treated group and $2400 \pm 510 \text{ mm}^3$ in the control group on day 20 after starting the treatment. TUNEL staining of tumours was performed to evaluate whether apoptosis was induced in BMA-treated xenograft tumours. A large number of tumour cells showed signs of apoptosis after BMA treatment, as confirmed by TUNEL staining (Figure 4E).

DISCUSSION

Prognostic significance of Bcl-xL in UTUC human samples. Apoptosis-related molecular markers are important for predicting the biological behavior of cancer cells, including UC cells. Deregulation of apoptosis-related proteins has been reported to have a central role in oncogenesis and cancer progression in bladder cancer (Shariat *et al*, 2004; Karam *et al*, 2007). The assessment of combined apoptosis markers could help identify those patients at high risk for disease recurrence and mortality.

The associations between apoptosis-related proteins, including p53, Bcl-2, Bax, and survivin, in UTUC have been previously reported. The molecular marker p53 is the most investigated marker in bladder carcinoma; however, there is insufficient evidence to conclude that p53 acts as a marker of outcome (Malats *et al*, 2005). In UTUC patients, several studies reported that overexpression of p53 is related to increased tumour proliferation and disease progression and survival (Rey *et al*, 1997; Zigeuner *et al*, 2004; Nakanishi *et al*, 2005). On the other hand, other studies reported that there was no relationship between p53 expression and the prognosis in UTUC patients (Fromont *et al*, 2005; Jeong *et al*, 2009). Therefore, whether the expression of p53 or the function has prognostic value in UTUC patients is still controversial. Bcl-2 was also investigated in several studies to determine whether it is a significant prognostic factor in UTUC patients. However, the results of these studies suggested that there

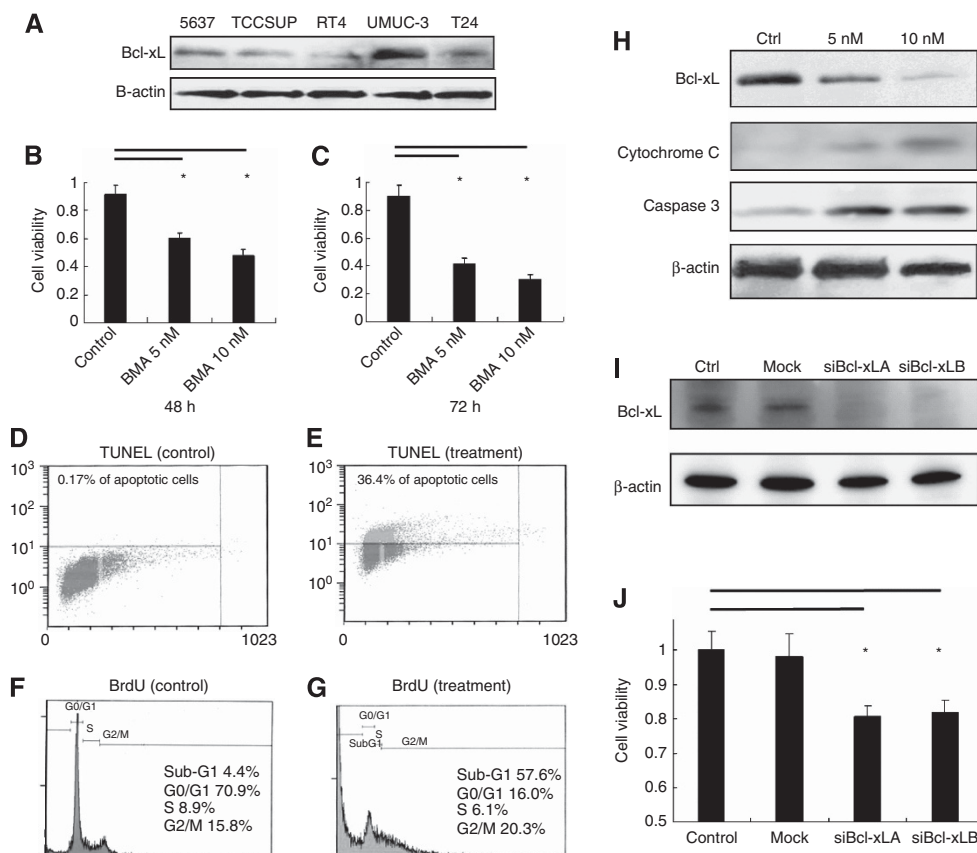


Figure 3. Targeting therapy for Bcl-xL *in vitro* study using BMA. (A) Western blot analyses of Bcl-xL expression in various bladder cancer cell lines. The expression level of bladder cancer cell lines (5637, TCCSUP, RT4, UMUC-3, and T24) with western blot analysis. (B, C) Cell growth inhibitory effects of BMA in UMUC-3 cells. The cells were treated with (B) 48-h and (C) 72-h exposure to 5 nM or 10 nM BMA and cell viability was measured by WST-1 assay. Cells treated with the same concentrations of DMSO served as controls. * $P < 0.01$ compared with control. (D–G) Detection of apoptosis by 5 nM BMA flow cytometric analyses were performed by TUNEL assay and BrdU assay to detect apoptotic cells in (D, F) vehicle control and (E, G) 5 nM BMA treatment, respectively. Upper left quadrants of each graph represent the apoptotic cell populations. Cells treated with the same concentrations of DMSO served as controls. (H) Western blot analyses on Bcl-xL, cytochrome c, and caspase-3 protein. Western blot analysis was performed using antibodies specific for Bcl-xL, cytochrome c, and caspase-3. Protein was prepared after exposure to various concentrations of BMA. Cells treated with the same concentrations of DMSO served as controls. (I) siRNA for Bcl-xL in UMUC-3 cells. Western blotting for Bcl-xL protein, 48 h after transfection with the concentration of 20 nM siBcl-xLA and siBcl-xLB, respectively. (J) Cell viability assay for UMUC-3 cells with 20 nM siBcl-xLA and siBcl-xLB 72 h after transfection was measured by WST-1 assay, * $P < 0.05$ compared with control.

was no significant association between Bcl-2 expression and the prognosis of UTUC patients (Nakanishi *et al*, 1998; Korkolopoulou *et al*, 2002; Jeong *et al*, 2009). Jeong *et al* (2009) evaluated the expression of Bax in UTUC specimens and reported that Bax expression was not associated with the clinical outcome of patients with UTUC. Survivin expression in UTUC tissues was also evaluated in several studies. However, the evaluation of survivin and its prognostic significance are controversial (Nakanishi *et al*, 2002; Jeong *et al*, 2009). Therefore, the prognostic significance of these apoptosis-related markers has not yet been fully characterised in patients with UTUC.

Bcl-xL is a pro-survival member of the Bcl-2 family and has an important role in regulating cell survival and apoptosis. Bcl-xL has been found to be overexpressed in many human cancers, and its prognostic role was investigated in several studies, the results of which suggest Bcl-xL overexpression may indicate a poor prognosis for patients (Watanabe *et al*, 2004; Williams *et al*, 2005; Karczmarek-Borowska *et al*, 2006; Soltani-Arabshahi *et al*, 2009). Recently, Jin-Song *et al* (2012) reported that the level of Bcl-xL protein expression was closely correlated with tumour characteristics in colorectal cancer patients, and patients with high Bcl-xL expression showed poorer overall survival than those with low Bcl-xL expression. Differences in the intensity of Bcl-xL

expression in tumour specimens may have an effect on the control of cancer cell death. Therefore, these data suggest that Bcl-xL expression or its intensity have an important role as a significant molecular marker in human malignancies. Bcl-xL expresses in high frequency in UC of urinary bladder; however, it is unclear whether there is a relationship between the prognosis of bladder cancer and the expression of Bcl-xL (Korkolopoulou *et al*, 2002). Taking into consideration such a result, we decided to evaluate the prognosis of UTUC patients using the expression level of Bcl-xL protein in tissue obtained from the patients.

In the present study, we investigated 175 cases of UTUC and analysed the significance of the expression of pro-survival Bcl-xL protein by immunohistochemistry. Our results indicated that a high Bcl-xL score in tumour specimens was an independent prognostic indicator for CSS in UTUC patients. When we analysed the clinicopathological data in the subgroup of patients with a higher pathological stage, higher tumour grade, or LVI positive, a high Bcl-xL score was significantly associated with a worse CSS. These results indicated that the deregulation of pro-survival pathways in UTUC is significantly associated with cancer progression and Bcl-xL may become a useful prognostic marker, which might lead to the identification of patients who stand to benefit from intensified therapy such as adjuvant chemotherapy

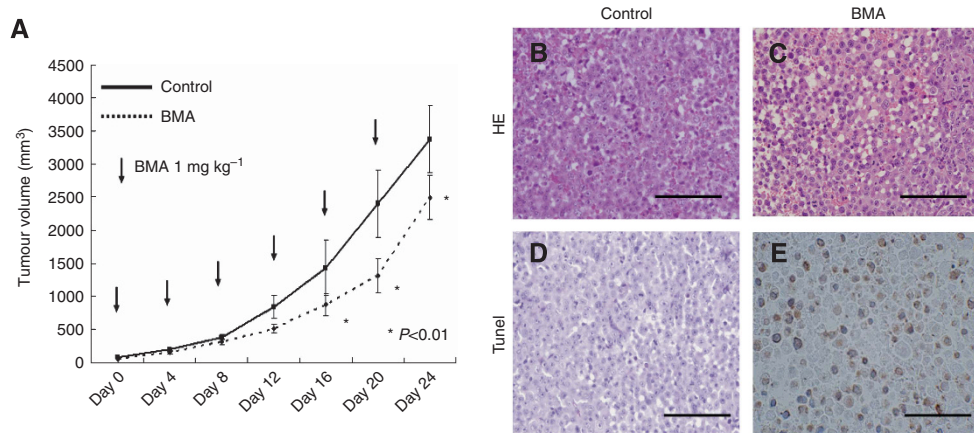


Figure 4. Efficacy of BMA on tumour growth in xenograft UMUC-3 tumour model. **(A)** UMUC-3 cells (1×10^6) were implanted subcutaneously into the flank of nude mice. When an animal developed a palpable tumour, it was given an intraperitoneal injection of 1 mg kg^{-1} of BMA or vehicle control every 4 days for 24 consecutive days. BMA was injected a total of six times in the treatment group. Tumour volume was monitored every 4 days. *Statistically significant difference compared with vehicle controls, $P < 0.01$. **(B–E)** Histopathological evaluation in BMA-treated UMUC-3 tumours. Hematoxylin–eosin (HE) staining of **(B)** UMUC-3 tumour treated by vehicle control and **(C)** that of tumour treated with 1 mg kg^{-1} of BMA. TUNEL staining of **(D)** UMUC-3 tumour treated by vehicle control and **(E)** tumour treated with BMA (bars = $100 \mu\text{m}$).

and might provide an appropriate monitoring schedule with appropriate follow-up. To the best of our knowledge, this is the first report to examine the prognostic significance of Bcl-xL expression in a large number of UTUC cases.

Therapeutic efficacy of targeting Bcl-xL using BMA *in vitro* and *in vivo*. Our clinicopathological results suggest that targeting Bcl-xL protein seems to be a reasonable approach for assembling therapeutic strategies for the treatment of UC patients to improve their prognosis. Using siRNA targeting Bcl-xL, we demonstrated anti-cancer effect in bladder cancer cells. Therefore Bcl-xL is to be one of the important molecules for bladder cancer cell survival. Kunze *et al* (2012) reported that simultaneous siRNA-mediated knockdown of antiapoptotic protein mediated a significant reduction in cell viability and induction of apoptosis, suggesting Bcl-xL itself can be targeted for treatment. Therefore, clinically achievable molecules targeting Bcl-xL are needed for cancer therapy. In the present study, we focused on V-ATPase inhibitors, which can disturb the anti-apoptotic function of Bcl-xL. BMA has demonstrated an anti-cancer effect in several malignancies (Ohta *et al*, 1998; Nakashima *et al*, 2003; Morimura *et al*, 2008) and been shown to regulate the function of Bcl-xL protein in cancer cells in lung cancer cell lines (Sasazawa *et al*, 2009). Sasazawa *et al* (2009) suggested that BMA, a potent functional suppressor of Bcl-xL, functions not as a small molecule that can inactivate by recognising the surface pocket of Bcl-xL but rather as a modulator of cellular pH which induces the alternation between pro- and anti-apoptotic protein function. The role of V-ATPase in cell death and the mechanisms of apoptosis in cancer cells are still not fully elucidated. Recently, von Schwarzenberg *et al* (2013) reported that V-ATPase inhibitors induce the energy starvation stress response with a decline in the ATP level in the cellular environment before induction of cell apoptosis. Dysfunction of V-ATPase induced by an inhibitor may affect intracellular homeostasis supported by an appropriate pH balance. Our experiments demonstrated that the Bcl-xL was an important molecule for cancer cell survival; however, mainly reported the effect of BMA treatment and did not investigate specifically whether BMA really targets only Bcl-xL protein. BMA, which is a potent inhibitor of V-ATPase, has several functions for cancer cells and its cell survival control. Therefore, the role as the Bcl-xL inhibitor may be one of the most important functions of BMA in cancer treatment. Further investigations are needed to clarify the interactions between BMA and Bcl-xL protein.

No study has evaluated whether targeting therapy for Bcl-xL proteins by BMA could induce apoptosis or whether it has an anti-tumour effect in UC cells. Our *in vitro* study demonstrated that BMA inhibited UMUC-3 cell proliferation by induction of apoptosis through a caspase-dependent pathway. *In vivo* study revealed that BMA significantly inhibited tumour growth compared with the control group. Ohta *et al* (1998) reported that the administration of BMA significantly inhibited tumour growth compared with control in nude mice bearing a xenografted pancreatic tumour cell line. Our data also demonstrate the effectiveness of xenograft tumour treatment with UC cells without any toxicity. Furthermore, TUNEL staining demonstrated that BMA induced apoptosis in UMUC-3 xenograft tumours *in vivo*, a finding that was compatible with the *in vitro* study results. We also analysed histologically the major organs, including liver, kidney, and spleen, by preparing HE-stained sections and found that there were no significant differences in the histological appearance or damage to these organs between the control and treated mice. However, it is still unknown how BMA regulates the expression of Bcl-xL protein in cancer cells, and continuous effort is needed to delineate the precise mechanisms of apoptosis caused by BMA *in vitro* and *in vivo*.

In conclusion, the findings of the present study indicate that Bcl-xL expression can predict the survival of UTUC patients. Therapy that targets Bcl-xL may be a promising therapeutic strategy for patients with UC.

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