RESEARCH ARTICLE

High Expression Level of Preoperative Serum Uroplakin III is Associated with Biologically Aggressive Bladder Cancer.

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Abstract

Background: Uroplakins have been widely investigated as potential markers in patients with bladder cancer because these proteins are specific to the urothelium. However, the role of uroplakin proteins in bladder cancer remains unknown. In this study, preoperative serum levels of uroplakin III were measured in patients with urothelial carcinoma of the urinary bladder and examined for possible association with clinicopathological features and clinical outcomes. Materials and Methods: This study included 52 bladder cancer patients at various stages and 28 healthy controls. Uroplakin III levels were detected in preoperative sera using an automated dot blot system and a micro-dot blot array. Results: There was a significant increase in serum uroplakin III levels in patients with bladder cancer as compared to healthy controls ($p<0.05$). In addition, serum uroplakin III levels were associated with muscle-invasive status, high grade and lymphovascular invasion ($p<0.02$). Log-rank tests indicated high serum uroplakin III to be significantly associated with cancer-specific mortality. Conclusions: Determination of serum uroplakin III level could be valuable for identifying patients with biologically aggressive bladder cancer.

Keywords: Urothelial carcinoma - bladder - serum marker - protein - uroplakin

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Introduction

Preoperative predictive models of disease progression and metastasis are essential to select the optimal treatment for patients with aggressive bladder cancer (BC). The ability to predict poor prognosis before initial treatment would enable physicians to select early intervention and/or intensive systemic treatment for better outcome (Ikeda et al., 2014; Otunctemur et al., 2014). Various predictive models have been widely investigated to reduce BC-related death. One of the challenges is precisely predicting the pathological stage, which is a reliable and established factor connected to disease prognosis (Wang et al., 2012; Ghafouri-Fard et al., 2014). While preoperative computed tomography and magnetic resonance imaging for BC staging are under development, their accuracy for predicting pathological stage varied between 40% and 90% (El-Assmy et al., 2009; Takeuchi et al., 2009). Although repeat transurethral resection (TUR) can help to stratify patients based on whether they have muscle-invasive cancer, pathologists may have difficulties diagnosing precise cancer stage and grade from tissues obtained by TUR because of the damage done to specimens by electric cautery (Schwaibold et al., 2006). To overcome these limitations, preoperative molecular markers are expected to be a minimally invasive method for predicting precise prognosis and progression in patients with BC.

Uroplakin plays a key role in urothelial functions, including participation in the permeability barrier, adjustment of urothelial surface area, stabilization of the urothelial surface and development of the urinary tract (Huang et al., 2007). Owing to their specific expression in the urothelium, uroplakin has been investigated as a potential immunohistochemical markers for the primary lesions and the identification of the primary cancer in patients with metastases of unknown origin (Xu et al., 2001). Uroplakin family comprised a group of 4 transmembrane proteins, including Ia (27kDa), Ib (28kDa), II (15kDa) and III (47kDa) (Wu et al., 1994). Uroplakin III, which is the largest mass in uroplakin family, is only applied as immunohistochemical marker in a clinical setting and we previously showed that the loss of expression of immunohistochemical staining for uroplakin III is associated with pathologically aggressive BC and poor prognosis in patients undergoing radical cystectomy (Matsumoto et al., 2008). However, the utility of serum uroplakin III, e.g. predictive models of disease outcome, in patients with BC is unknown. In the present study,

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we hypothesized that damage to the urothelial surface caused by aggressive tumor growth releases uroplakin III into the blood vessels and quantitative analysis of this urothelium-specific protein in the serum might have the potential to be used as preoperative predictive models of prognosis in patients with BC. The primary objective was whether the circulating levels of preoperative uroplakin III is associated with pathological features and clinical outcomes in patients with clinical TanyN0M0. The secondary objective was to investigate the possibility for the circulating uroplakin III level to be used as a potential diagnostic biomarker for BC compared with healthy controls.

Materials and Methods

Patient population

Candidates for this study were 52 patients with urothelial carcinoma of the urinary bladder who were treated at our institution between august 2004 and july 2009. Twenty-two of these patients were treated with radical cystectomy and bilateral pelvic lymphadectomy and the other 30 were treated with TUR. Preoperative serum levels of uroplakin III were measured. Routine laboratory studies, chest X-ray and pelvic computed tomography or magnetic resonance imaging were performed and no evidence of clinically distant or lymph node metastasis was found in any of the patients. There were 44 men (83%) and 8 women (17%) with a median age of 70 years (range 39-82). BCs were staged according to the TNM system (2002) and the World Health Organization classification (1973) was used for grading. Pathological stage of the BCs was Ta in 7 cases (14%), T1 in 21 (40%), T2 in 8 (15%), T3 in 10 (19%) and T4 in 6 (12%). A postoperative follow-up examination was scheduled every 3 and 4 months following TUR and cystectomy, respectively, for the first year. Semiannual examinations were done in the second year, with annual examinations thereafter; more frequent examinations were scheduled if clinically indicated. Median follow-up from the procedures was 52 months (range 6-98). None of the patients had prior radiation or systemic chemotherapy before surgery.

We also measured serum uroplakin III levels in 28 controls from healthy volunteers. Approval was granted by the ethics committee of Kitasato university school of medicine and hospital and all patients signed written informed consent.

Measurement of serum uroplakin III

All serum samples were kept at -80°C until use. Serum uroplakin III levels were detected by using an automated micro-dot blot array with a 256 solid-pin system (Kakengeneqs Co., Ltd., Chiba, Japan). In brief, the removal of albumin and IgG from sera was performed using a ProteoExtract Albumin/IgG removal kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions (Kobayashi et al., 2012). 1 μl each of 20-times diluted albumin- and IgG-depleted sera was spotted onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA). The membranes were then blocked with 20% N101 (NOF Corp., Tokyo, Japan)/TBS for 1 h at room temperature. After being washed in TBS, the membranes were reacted with 100-times diluted primary monoclonal antibody against uroplakin III (ready to use, Nichirei, Tokyo, Japan) with 1% N101/TBS for 30 min at room temperature. After being washed with TBS containing 0.1% Tween-20, the membranes were incubated with 1,000-times diluted horseradish peroxidase-conjugated anti-mouse IgG polyclonal antibody for 30 min at room temperature. Finally, signals were developed with Immobilon Western reagent (Millipore Corp.). The data were analyzed using DotBlotChip-System software ver. 4.0 (Dynacom Co., Ltd., Chiba, Japan). Normalized signals are presented as the positive intensity minus background intensity around the spot.

Statistical analysis

For the purpose of analysis, gender, age (less than 65 versus 65 and greater), pathological stage (Ta and 1 as non-muscle invasive bladder cancer [NMIBC] versus T2, 3 and 4 as muscle invasive bladder cancer [MIBC]), pathological grade (grade 1 and 2 versus grade 3), lymphovascular invasion and lymph node metastases were evaluated as dichotomous variables. Unpaired t-test was used to compare the serum uroplakin III levels between healthy controls and BC patients. Unpaired t-test was also used to evaluate the association of the serum uroplakin III levels with clinicopathological characteristics. The Kaplan Meier method was used to calculate survival functions and differences were assessed with the log rank statistic. Multivariable survival analyses were performed with the Cox proportional hazards regression model, controlling for pathological stage and grade, lymphovascular invasion, lymph node metastases and preoperative serum uroplakin III level. The area under the curve (AUC) and best cut-off point were calculated employing the receiver-operating characteristic (ROC) analysis. $p<0.05$ was considered to indicate a statistically significant difference. All the reported p values were two sided. Analyses were performed using SPSS, version 11.0 for windows (SPSS, Inc., Chicago, IL, USA), GraphPad prism, version 5 (GraphPad Software, Inc., CA, USA) and microsoft excel (Microsoft, Redmond, WA, USA).

Results

Validation of preoperative serum uroplakin III

To evaluate the utility of the preoperative serum uroplakin III levels as a potential diagnostic biomarker for BC, we investigated serum uroplakin III levels in patients with BC and healthy controls by dot blot analysis. The median level of serum uroplakin III in patients with NMIBC, in those with MIBC and in healthy controls was 1.262 (range 0-6.851), 2.776 (range 0.658-6.032) and 0.678 (range 0-2.739), respectively (Figure 1). There was significantly greater increase of serum uroplakin III level in patients with NMIBC and MIBC than in healthy controls ($p=0.040$ and $p<0.001$, respectively). ROC curve analysis of serum uroplakin III level was performed for the comparison of BC groups with the control one. The AUC-ROC level for NMIBC and MIBC was 0.62 (95%CI:

0.47-0.77, p=0.110, Figure 2A) and 0.88 (95%CI: 0.79-0.97, p<0.001, Figure 2B), respectively. The sensitivity and specificity in NMIBC, using a cut-point of 2.115, were 28.5% (95%CI: 0.132-0.486) and 96.4% (95%CI: 0.816-0.999), respectively. The sensitivity and specificity in MIBC, using a cut-point of 2.013, were 66.6% (95%CI: 0.446-0.843) and 96.4% (95%CI: 0.816-0.999), respectively.

Association of preoperative serum uroplakin III with clinicopathological characteristics

Table 1 showed the association of preoperative serum uroplakin III with clinicopathological features in patients with BC. Preoperative serum uroplakin III levels were significantly higher in patients with MIBC than in those with NMIBC (p=0.003). Patients with positive versus negative lymphovascular invasion had significantly different in preoperative serum uroplakin III (2.489 versus 1.276, p=0.015). Preoperative serum uroplakin III levels were also significantly higher in patients with pathological grade 3 disease than in those with grade 1 and 2 diseases (2.489 versus 1.260, p=0.005). There were no significant differences in serum uroplakin III levels in terms of gender, age, lymph node status.

Association of serum UP III levels with clinical outcomes

Of 52 patients, 12 (23%) had succumbed to BC at the time of analysis (median time to death: 26 months; range: 8-74 months). The log-rank test results indicated that the normalized signals from patients with serum uroplakin III above 1.7 had a significantly increased probability of BC-specific mortality (p=0.036, Figure 3). On multivariate Cox proportional hazards regression analysis, none of the factors was associated with an increased risk for cancer-specific mortality.

Table 1. Association of Preoperative Serum Uroplakin III with Clinical and Pathological Characteristics of Patients with Urothelial Carcinoma of The Urinary Bladder.

	No. of patients (%)	Serum uroplakin III level		p value
		Median	Range	
Gender				0.406
Male	44 (85)	1.836	0.000-6.851	
Female	8 (15)	1.706	0.020-3.227	
Age (years)				0.716
<65	18 (35)	1.599	0.000-6.851	
≥65	34 (65)	1.753	0.000-6.032	
Pathological stage				0.003
Ta or T1	28 (54)	1.262	0.658-6.032	
T2,3 or 4	24 (46)	2.776	0.000-6.851	
Lymphovascular invasion				0.015
Positive	35 (67)	1.276	0.000-6.851	
Negative	17 (33)	2.489	0.658-6.032	
Lymph node metastases				0.394
N0	46 (88)	1.661	0.000-6.851	
N1, N2	6 (12)	2.627	0.737-5.438	
Pathological grade				0.005
Grades 1 and 2	27 (52)	1.26	0 000-5.389	
Grade 3	25 (48)	2.489	0.246-6.851	

Discussion

In the present study, we assessed the preoperative serum uroplakin III levels in patients with clinical TanyN0M0 by using a micro-dot blot system and the association with pathological features and clinical outcomes. We found that high level of uroplakin III in the serum was associated with poor prognosis and biologically aggressive factors including muscle invasive lesion, high-grade and the status of lymphovascular invasion. These findings suggest that this urothelium-specific protein has the potential to be the basis for a predictive model of prognosis in patients with BC. Although none of the molecular markers are clinically used for determining prognosis, reliable molecular markers that can predict cancer survival will be able to avoid excessive chemotherapy and select patients

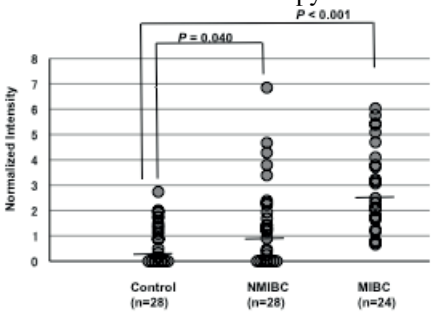

Figure 1. Serum Uroplakin III Level in Healthy Controls and Bladder Cancers by Dot Blot Analysis. Horizontal lines indicate medians. NMIBC: non-muscle invasive bladder cancer; MIBC: muscle invasive bladder cancer.

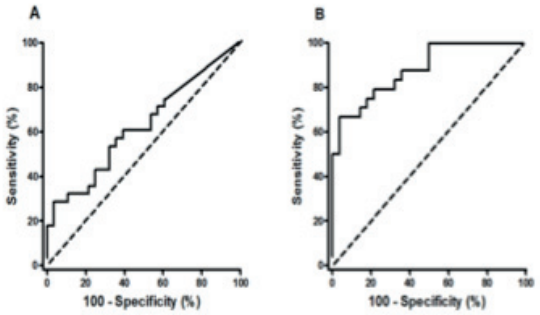

Figure 2. The Receiving-Operating Characteristic (ROC) Analysis of Preoperative Serum Uroplakin III Level to Detect Bladder Cancer. Diagnosis of non-muscle invasive bladder cancers versus healthy controls (the area under the curve (AUC) 0.62, 95% CI 0.47-0.77, p=0.110) (A). Diagnosis of muscle invasive bladder cancers versus healthy controls (AUC 0.88, 95% CI 0.79-0.97, p<0.001) (B).

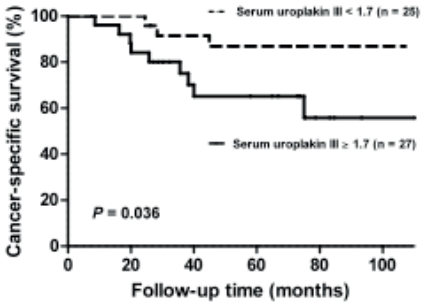

Figure 3. Probability of Cancer-Specific Survival After Surgical Treatment According to The Serum Uroplakin III Level.

who need intensive treatment. Physicians may have to select intensive systemic treatment before surgery, which has been demonstrated to have a clear survival benefit in patients with clinical T2-4N0M0 (Griffiths et al., 2011), because they are unlikely to be cured by only surgery.

Other investigators have evaluated the role of the status of uroplakin in blood sample in patients with BC (Li et al., 1999; Lu et al., 2000). Lu et al (Lu et al., 2000) detected circulating uroplakin II mRNA-positive cells by nested reverse-transcription PCR assay. The detection rate in patients with muscle invasion, regional lymph-node metastases and distant metastases was 28.6%, 40.0% and 75%, respectively. The positive rates were increased with tumor extension. Li et al (Li et al., 1999) also performed sequential blood sampling in two patients who underwent systemic chemotherapy for metastatic diseases. These two patients responded well to chemotherapy and their uroplakin II-positive cells disappeared during several cycles of chemotherapy. That study showed uroplakin II in peripheral blood might be used as a tumor marker for molecular staging and treatment response.

Previous studies conducted our group showed that the loss of expression of immunohistochemical staining of uroplakin III is associated with established markers of aggressive BC including high stage, high grade and lymphovascular invasion and decreased cancer-specific survival in patients undergoing radical cystectomy (Matsumoto et al., 2008). Those results are discordant with the present study. The increased levels of preoperative serum uroplakin III are associated with poor prognosis and pathologically aggressive factors. Uroplakin III is normally expressed on normal urothelium. Aggressive BC could damage the normal urothelium and cause the loss of expression of immunohistochemical staining for uroplakin III. This serum protein might be leaked from the normal urothelium damaged by aggressive cancer. More aggressive cancer may damage normal urothelium more forcefully and leak this protein into blood more frequently. We speculate that increased levels of this serum protein would not be mediated by excessive production from aggressive cancer cells because similar phenomenon was demonstrated the status of E-cadherin, which has been known as an intercellular adhesion molecule. Expression of E-cadherin was increased in serum sample, but was decreased or lost in immunohistochemical staining in patients with aggressive BC (Bringuier et al., 1993; Matsumoto et al., 2003). A regulated mechanism of the cell membrane proteins such as uroplakin III and E-cadherin is similar in aggressive BC, however, the relationship between expression of immunohistochemical staining and serum levels of cell membrane proteins is not well understood.

While prostate-specific antigen (PSA) test is utilized in the detection of prostate cancer, a diagnosis of BC relies on imaging modality and cystoscopy because effective and simple screening biomarkers are lacking. In this study, we investigated the possibility for preoperative serum uroplakin III level and found that serum uroplakin III level was higher in patients with BC than those in healthy controls. In addition, preoperative serum uroplakin III showed high specificity (96%) in patients with NMIBC and high sensitivity (66%) in patients with MIBC. These results indicate that preoperative serum uroplakin III has the potential to become an additional diagnostic tool for BC. On the other hand, preoperative serum uroplakin III had low sensitivity for the detection of NMIBC (33%). Abnormal serum uroplakin III level could not occur at an early stage of BC. One possible reason is that NMIBC do not invade muscle layer which is rich in blood and lymphatic vessels.

This study had limitations. First, any parameters were not associated with cancer-specific mortality on multivariate analysis because of the limited number of cases. A prospective study involving more patients is needed to clarify the prognostic value of preoperative serum uroplakin III. Second, abnormal serum uroplakin III level did not occur in all cases of BCs, especially at early stage. Therefore, we did not investigate other possible reasons for the high expression of serum uroplakin III related to benign diseases, including inflammation, hydronephrosis and urolithiasis.

Despite these limitations, serum uroplakin III was higher in patients with BC than those in healthy controls. In addition, high level of preoperative serum uroplakin III was associated with established features of biologically aggressive BC such as pathological stage and grade and lymphovascular invasion. This would help physicians make decisions regarding individual treatment. Expression level of serum uroplakin III could be detective and predictive marker for patients who are at increased risk of worse prognosis. Further research is warranted to clarify the availability and boundary of serum expression of uroplakin III in patients with BC.

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