

RESEARCH ARTICLE

Serum Periplakin as a Potential Biomarker for Urothelial Carcinoma of the Urinary Bladder

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Abstract

The objectives of this study were to examine serum periplakin expression in patients with urothelial carcinoma of the urinary bladder and in normal controls, and to examine relationships with clinicopathological findings. Detection of serum periplakin was performed in 50 patients and 30 normal controls with anti-periplakin antibodies using the automatic dot blot system, and a micro-dot blot array with a 256 solid-pin system. Levels in patients with urothelial carcinoma of the urinary bladder were significantly lower than those in normal controls (0.31 and 5.68, respectively; $p < 0.0001$). The area under the receiver-operator curve level for urothelial carcinoma of the urinary bladder was 0.845. The sensitivity and specificity, using a cut-off point of 4.045, were 83.7% and 73.3%, respectively. In addition, serum periplakin levels were significantly higher in patients with muscle-invasive cancer than in those with nonmuscle-invasive cancer ($P = 0.03$). In multivariate Cox proportional hazards regression analysis, none of the clinicopathological factors was associated with an increased risk for progression and cancer-specific survival. Examination of the serum periplakin level may play a role as a non-invasive diagnostic modality to aid urine cytology and cystoscopy.

Keywords: Periplakin - urothelial carcinoma - diagnosis

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Introduction

Urothelial carcinoma of the urinary bladder (UCB) is the second most common malignancy in the genitourinary tract. Approximately 75% of UCB cases are diagnosed as nonmuscle-invasive bladder cancer (NMIBC) at the first diagnosis (Shelley et al., 2010). NMIBC has the tendency to recur and may progress to muscle-invasive bladder cancer (MIBC), which is a life-threatening neoplasm (Ikeda et al., 2014). Cystoscopy and urine cytology are typical modalities for the diagnosis and surveillance of UCB. Cystoscopy can identify the most papillary and solid lesions, but it is physically uncomfortable for patients. Use of urine cytology is limited because of its low sensitivity. For these reasons, some tumor markers have been investigated (eg, BTAstat, NMP22), but their sensitivity and specificity are limited and not superior to urine cytology (Toma et al., 2004). To overcome these limitations, preoperative molecular markers are expected to be used as a minimally invasive method for assisting in and predicting a precise diagnosis in patients with UCB (Ghafouri-Fard et al., 2014).

The plakin family mediates the tissue filaments that represent the cell cytoskeleton in cell-to-cell junctions mediated by cadherin, and it is able to withstand mechanical stimulation and provide integrity of tissues (Jefferson et al., 2004; Sonnenberg et al., 2007). Dysfunctional plakin proteins contribute to diverse diseases, and autoantibodies and mutations perturb their activities with profound consequences. Seven plakin proteins are found in mammalian cells. Envoplakin, desmoplakin, and periplakin are associated with desmosomes in various solid tissues. A proteomics technique including two-dimensional gel electrophoresis (2-DE) combined with immunoblot analysis has been shown to identify tumor-associated antigenic proteins for UCB (Minami et al., 2014). Periplakin is a candidate for being a tumor marker in patients with UCB. The 195-kDa membrane-associated protein periplakin is involved in cellular movement and attachment (Nagata et al., 2001).

We previously found that loss of periplakin expression was associated with biological aggressiveness of UCB using immunohistochemical staining (Matsumoto et al., 2014). In addition, the majority of UCB showed loss or

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decreased expression patterns compared with normal or benign lesions on pathological slides. Next, we sought to determine whether the dynamics of serum periplakin would detect UCB and predict the prognosis in patients with UCB.

The primary objective of this study was to investigate the circulating periplakin levels needed for use as a potential detection marker for UCB. The secondary objective was to determine whether the levels of periplakin would be associated with clinicopathological features and prognosis in patients with UCB.

Materials and Methods

Patients

This retrospective study included 52 patients with UCB who were treated at Kitasato University Hospital between August 2004 and July 2009. Serum samples from two patients (a man and a woman) were used in other studies (Tsumura *et al.*, 2014). There were 43 men (86%) and 7 women (14%) with a median age of 70 years (mean=68.5; range=39–82 years). Twenty-two of these patients were treated with radical cystectomy and bilateral pelvic lymphadenectomy, and the other 28 were treated with transurethral resection (TUR). Preoperative serum levels of periplakin were measured. Laboratory studies, chest X-ray, and pelvic computed tomography or magnetic resonance imaging were routinely investigated, and there was no evidence of clinical distant or lymph node metastasis in any of the patients. The 2002 Tumor–Node–Metastasis (TNM) classification was used for pathological staging, and the World Health Organization classification was used for pathological grading. Lymphovascular invasion (LVI) determined the presence of cancer cells within the endothelial space. Cancer cells that merely invaded a vascular lumen were considered negative.

The median follow-up time was 63.3 months (mean=60.9; range=6.4–125.9 months) for those patients who were still alive at the last follow-up session. A postoperative follow-up examination was scheduled every 3 to 4 months after TUR and cystectomy, respectively, during the first year. Semi-annual examinations were performed during the second year, with annual examinations thereafter. More frequent examinations were scheduled if clinically indicated. None of the patients had previous radiation or systemic chemotherapy before surgical treatment, and none had a history of pulmonary or skin diseases.

We also measured serum periplakin levels in 30 normal controls (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 periplakin

All serum samples were kept at -80°C until use. Serum periplakin 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; 1 µl each of 20-times diluted albumin-depleted and IgG-depleted sera was spotted onto polyvinylidene difluoride membranes (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 polyclonal antibody against periplakin (Santa Cruz Biotech, Dallas, TX, USA) 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 1000-times diluted horseradish peroxidase-conjugated anti-mouse IgG polyclonal antibody (Dako, Glostrup, Denmark) for 30 min at room temperature. Finally, signals were developed with Immobilon Western reagent (Millipore Corp.). The data were analyzed using DotBlotChip System software version 4.0 (Dynacom Co., Ltd., Chiba, Japan). Normalized signals are presented as the positive intensity minus background intensity around the spot.

Statistical analyses

For the purposes of our analysis, gender, age (younger than 65 versus 65 or older), pathological stage (Ta or T1 as NMIBC versus T2 or greater as MIBC), pathological grade (grades 1 and 2 versus grade 3), LVI (positive versus negative), and lymph node status (N0 versus N1 and N2) were evaluated as dichotomized variables. Mann-Whitney U test was used to evaluate the association of periplakin with gender, age, pathological stage and grade, lymph node status, and LVI. Mann-Whitney U test was also used to compare the serum periplakin levels between UCB patients and normal controls. The Kaplan Meier method was used to calculate survival functions, and differences were assessed with the log rank test. The area under the curve (AUC) and best cut-off point were calculated using the receiver-operating characteristic (ROC) analysis. Multivariate survival analyses were performed with the Cox proportional hazards regression model, controlling for serum periplakin, pathological stage and grade, presence of LVI, and lymph node metastases. Statistical significance was set as $p < 0.05$. All analyses were performed with StatView (version 5.0; SAS Institute, Cary, NC, USA).

Results

Validation of preoperative serum periplakin

The median levels of serum periplakin in patients with UCB and in normal controls were 0.31 (mean=1.96; range=0–20.49) and 5.68 (mean=6.11; range=0–17.59), respectively (Figure 1). There were significantly decreased serum periplakin levels in patients with UCB than in normal controls ($p < 0.0001$).

ROC curve analysis of serum periplakin level was performed for the comparison between the UCB group and the control group. The AUC-ROC level for UCB was 0.845 (95% CI=0.752–0.937) (Figure 2). The sensitivity and specificity in UCB, using a cut-off point of 4.045, were 83.7% (95% CI=70.3%–92.7%) and 73.3% (95% CI=54.1%–87.7%), respectively.

Association of preoperative serum periplakin with clinicopathological characteristics

The association of serum periplakin with clinicopathological features is shown in Table 1. Median serum periplakin levels in patients with NMIBC and MIBC were 0.00 and 1.48, respectively. Preoperative serum periplakin levels were significantly higher in patients with MIBC than in patients with NMIBC ($p=0.03$). There were no significant differences in serum periplakin levels in terms of gender, age, pathological grade, LVI, and lymph node status ($p>0.05$).

Association of periplakin expression with prognosis

Disease progression was observed in 13 patients (26%) (median time to progression=25.4; mean=27.4; range=4.8–64.8 months). Twelve patients (24%) (median time to death=29; mean=33.7; range= 9.2–80.4 months) died during the study period.

The Kaplan Meier method using the log-rank test indicated that the normalized signals from patients with serum periplakin above the median level of 0.31 showed no significant differences in terms of progression and cancer-specific survival.

In multivariate Cox proportional hazards regression analysis controlling for preoperative serum periplakin levels, pathological stage and grade, LVI, and lymph

Table 1. Association of Preoperative Serum Periplakin Levels with Clinical and Pathological Characteristics

	Total no.of patients (%)	Serum periplakin levels			P*
		Median	Mean	Range	
Gender					>0.99
Male	43 (86)	0.23	2.11	0.00-4.38	
Female	7 (14)	0.32	1.06	0.00-20.49	
Age (years)					0.41
<65	16 (32)	0	1.58	0.00-6.99	
≥65	34 (68)	0.51	2.14	0.00-20.49	
Pathological stage					0.03
pTa, pT1	27 (54)	0	1.15	0.00-4.08	
pT2, pT3, pT4	23 (46)	1.48	2.91	0.00-20.49	
Pathological grade					0.42
Grade 1, 2	26 (52)	0	1.7	0.00-7.88	
Grade 3	24 (48)	0.98	2.24	0.00-20.49	
LVI status					0.36
Negative	33 (67)	0.043	1.51	0.00-6.99	
Positive	17 (33)	0.74	2.85	0.00-20.49	
Lymph node status					0.44
Negative	44 (88)	0.5	2.12	0.00-3.75	
Positive	6 (12)	0.16	0.8	0.00-20.49	

*No: number; LVI: lymphovascular invasion. *Mann-Whitney U test

node status as dichotomous variables, none of the factors was associated with an increased risk for progression or cancer-specific survival.

Discussion

UCB ranks in the top category of newly diagnosed cancers. High-risk disease of NMIBC revealed high rates (up to 90%) of recurrence (Shelley et al., 2010). It is important to diagnose UCB accurately and quickly with the help of a simple and cost-effective method. Although TUR and histological examination remain the gold standard, urine cytology is helpful as a noninvasive method of early diagnosis of UCB (Matsumoto et al., 2014). With the currently available modalities, there is no reliable biochemical or molecular examination that could be used as a universal screening tool for UCB.

Although investigations of autoantibodies to periplakin were performed in several reports (such as those involving pulmonary and skin diseases) (Park et al., 2006; Taille et al., 2011), this is the first study to evaluate serum periplakin for cancer detection, particularly UCB. Serum periplakin was significantly lower in patients with UCB than in normal controls. In addition, using the best cut-off point determined by the ROC curve, preoperative serum periplakin potentially acts as a biomarker for diagnosis. With encouraging results using the dot plot system in regard to serum periplakin, the diagnosis of UCB could become more simple and noninvasive.

Recent studies reported the biological role of periplakin in cancerous lesions. Downregulation of periplakin was correlated with the progression of esophageal squamous cell carcinoma (Hatakeyama et al., 2006; Nishimori et al., 2006). Cyclin A2-induced upregulation of periplakin was associated with poor prognosis as well as cisplatin resistance in endometrial cancer cells (Suzuki et al., 2010). Periplakin silencing reduced migration and attachment of pharyngeal squamous cancer cells (Tonoike et al., 2011). Periplakin silencing in triple-negative breast cancer cells

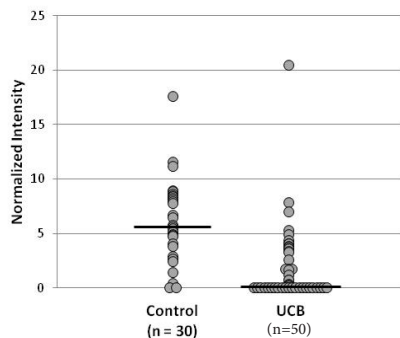


Figure 1. Serum Periplakin Expression Level in Normal Controls (n=30) and in those with Bladder Cancer (n=50) by Dot Blot Analysis. Horizontal lines indicate medians

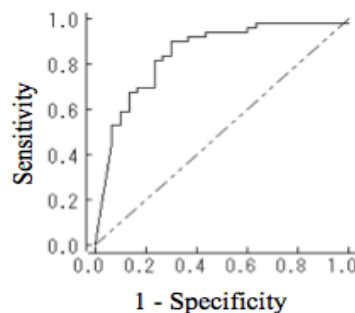


Figure 2. Receiver-Operating Characteristic (ROC) Analysis of Serum Periplakin Expression Level. The area under the curve-ROC level for bladder cancer was 0.845. The sensitivity and specificity, using a cut-off point of 4.045, were 83.7% and 73.3%, respectively

increased cell growth and reduced cell motility (Choi et al., 2013). We previously reported loss of periplakin expression was associated with pathological stage and cancer-specific survival in patients with UCB using immunohistochemical staining (Matsumoto et al., 2014). Periplakin is imperative for maintaining epithelial cell barriers, cellular movement, and attachment in normal physiology (Nagata et al., 2001; Jefferson et al., 2004; Sonnenberg et al., 2007). Normal expression of periplakin would suppress tumor progression; however, when altered, it would allow cancer cells to grow, detach, invade, and gain access to vascular and lymphatic systems.

Overexpression of cyclin A2 has been reported in various malignant tumors, including UCB (Chao et al., 1998; Dobashi et al., 1998; Mrena et al., 2006; Sun et al., 2011). Cyclin A2-induced cisplatin resistance is reflected by the suppression of drug-induced apoptosis. The cyclin A2-mediated reduction in apoptosis was attributable to activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, which is a major constituent of the mitochondrial anti-apoptotic pathway. The activation of the p-Akt pathway is reportedly one of the mechanisms by which carcinoma cells avoid the effect of chemotherapeutic drugs (Clark et al., 2002; Yang et al., 2006). p-Akt levels were elevated in cisplatin-resistant cells harboring PTEN gene mutation (Gagnon et al., 2004). Because periplakin is known to bind to intermediate filaments and Akt (van den Heuvel et al., 2002), Suzuki et al (Suzuki et al., 2010) showed a direct association of periplakin with Akt. Silencing of periplakin using siRNA significantly reduced basal p-Akt expression, suggesting that the binding of periplakin positively regulated the Akt activity. Interestingly, high expression of p-Akt was associated with high grade and stage of UCB (Sun et al., 2011), and locally advanced or metastatic UCB revealed resistance to cisplatin-based chemotherapy (Ikeda et al., 2011). We found that serum periplakin expression was higher in patients with MIBC than in those with NMIBC, but serum levels in both lesions were significantly decreased compared with that in normal controls. Although previous reports have shown that loss of expression of periplakin associated with pathological stage was seemingly discordant with the present study, both histological and serum expressions of periplakin indicated no or very slight expression in patients with UCB compared with normal controls. Although the precise role of serum periplakin protein in patients with UCB remains unknown, it is possible that biologically aggressive UCB could slightly increase the periplakin protein in serum concordant with the p-Akt pathway, but not to a high level, like that found under normal conditions, and then could progress locally or distantly and survive in anti-cancerous circumstances, for example, floating chemotherapeutic agents in the blood stream.

This study has several limitations. First, the relatively low number of patients did not allow us to show statistical power. Second, this study was performed exclusively in Japan, so more patients of different ethnicities and from countries with different genetic, epigenetic, and environmental risk factors are needed to confirm our results because the proteins of the plakin family are

detected as autoantibodies in other lesions (Park et al., 2006; Taille et al., 2011). Third, periplakin is located not only in urothelium but also in other cells. The role of serum periplakin expression needs to be validated in other types of diseases, including cancerous and inflammatory lesions. Finally, the detailed mechanisms and dynamics of periplakin between immunohistochemical staining and serum findings need to be determined.

In conclusion, patients with UCB demonstrated significantly decreased expression of serum periplakin protein compared with normal controls. In addition, we found that serum periplakin expression was higher in patients with MIBC than in those with NMIBC, but serum levels in both lesions were significantly decreased compared with those in normal controls. Further multi-institutional evaluations of serum periplakin in a large patient population are warranted before it can be included in routine clinical use for early detection of UCB. It may be suitable as an adjunct urine cytology and cystoscopy along with a noninvasive diagnostic modality.

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