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Original Article

Decreased expression of uroplakin Ia is associated with colorectal cancer progression and poor survival of patients

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Abstract: Aim: The present study was to investigate the clinical significance of Uroplakins Ia (UPKIa) in the development of colorectal cancer. Methods: mRNA levels of UPKIa in paired colorectal cancer lesions and the adjacent noncancerous tissues were examined using real-time PCR. The expression and prognostic value of UPKIa were examined in 125 colorectal cancer patients after resection. Statistical analyses were applied to derive prognostic associations. Results: UPKIa mRNA level was down-regulated in colorectal cancer lesions compared with that in the paired adjacent noncancerous tissues. Reduced expression of UPKIa was significantly associated with clinical staging ($P = 0.038$), and tumor size ($P = 0.035$) of the disease. Moreover, low expression of UPKIa was significantly associated with poorer overall (OS) and recurrent free (RFS) survival ($P = 0.017$ and $P = 0.007$, respectively) of colorectal cancer patients. Multivariate analysis suggested that reduced expression of UPK1a was an independent prognostic marker of colorectal cancer ($P = 0.047$). Conclusions: Low expression of UPKIa was a promising predictor for poor outcome of colorectal cancer patients. Further studies on the potential use of UPKIa as a therapeutic targetis are still needed.

Keywords: UPKIa, colorectal cancer, survival

Introduction

Colorectal cancer, one of the most aggressive carcinomas of the gastrointestinal tract, is a major cause of cancer-related deaths around the world [1]. It has been reported that the 5-year prevalence was estimated at 3,260,000, and the mortality of colorectal cancer was more than 600,000 deaths every year worldwide [2]. Although many improvements have been made in early tumor detection, surgical techniques and adjuvant therapy (chemotherapy and radiotherapy), distant metastasis and tumor recurrence substantial problems, and the outcomes of colorectal cancer remains far from satisfactory. Therefore, it is of great clinical value to find useful and specific biomarkers for the diagnosis and prognosis of the disease, as well as novel therapeutic strategies.

Uroplakins are urothelial differentiation-related membrane proteins that exhibited typical 2D crystals of hexagonally packed 16-nm particles. Initially, four major uroplakin proteins have been identified as uroplakins Ia, Ib, II and IIIa [3]. These proteins represent major urothelial cytodifferentiation products and are highly conserved during mammalian evolution [4]. The expression of uroplakins is paired, tetraspanins UPIa and UPIb interact with UPII and UPIII, respectively, and the formed heterodimers is a prerequisite for uroplakins to exit from the endoplasmic reticulum, and that co-transfection of correct uroplakin pairs significantly increase the stability of uroplakin proteins [5, 6]. Uroplakins Ia (UPKIa) and Ib belongs to a family of membrane proteins called 'tetraspanins', which have four transmembrane domains, a small and a large extracellular loops, and several highly conserved amino acid residues including 4-6 cysteines and several polar residues in or near the transmembrane domains [7]. Some tetraspanin genes have been shown to have reduced patterns of expression in cancer, suggesting that they may function as tumor suppressor genes [8]. Recently, UPKIa has

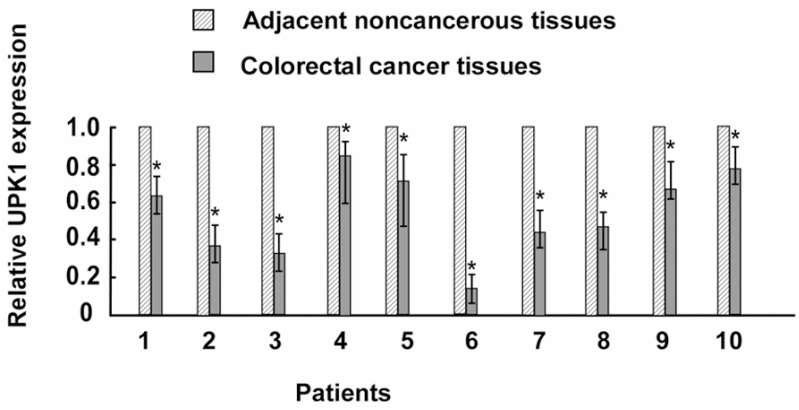

Figure 1. Decreased UPKIa mRNA expression in colorectal cancer specimens was detected by Real-time PCR (n = 10) compared with adjacent non-tumor tissue. asterisks, $P < 0.05$.

been implicated as a prognostic biomarker in several tumors. Kong et al. reported that UPKIa expression was down-regulated in esophageal squamous cell carcinoma (ESCC), and that ectopic expression of UPKIa in ESCC cells inhibited cell proliferation, suggesting that UPKIa serve as an important tumor suppressor gene in ESCC progression [9]. Zheng et al. demonstrated that decreased UPKIa expression is associated with poor clinical prognosis of gastric adenocarcinoma patients [10]. However, the clinicopathological and prognostic significance of UPKIa in colorectal carcinoma has not yet been described.

In this study, we report for the first time characterization of UPKIa expression in human colorectal cancer tissues and their correlation with clinicopathologic features. We found that UPKIa expression was correlated with clinical staging and tumor size of the disease. Multivariate analysis suggested that UPKIa expression was an independent prognostic marker for overall and recurrent-free survival in patients with colorectal cancer. Our data suggest that UPKIa might play a role in the development and progression of colorectal carcinoma, and it might be a potential prognostic biomarker for predicting postoperative survival in patients with colorectal carcinoma.

Materials and methods

Patients and tissue specimens

Clinical tissue samples used in this study were clinically and histologically diagnosed at the ***** from 2005 to 2009. For the use of these clinical materials for research purposes, prior patient's consent and approval from the Institute Research Ethics Committee was obtained. The Dukes classification was used for pathologic slides, and the World Health Organization classification was used for pathologic grading. None of the patients had undergone either chemotherapy or radiotherapy before the collection of the samples. Patients were followed-up every 3 months from the time of primary resection for the first two years, followed by every 6 months for five years.

Real time PCR analyses

Total RNA from fresh tissues were extracted using a Trizol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was treated with DNase, and 2.ug of total RNA was used for cDNA synthesis using random hexamers. For the evaluation of the relationship between GAPDH (internal control) and UPKIa, the primer selected were as follows: for UPKIa, forward, 5'-TGCCATCTTCTGCGGCTTCT-3', reverse, 5'-ATCACGGTGGGTGTAGGACG-3'; and for GAPDH, forward, 5'-CTCCTCCTGTTCGACAGTCAGC-3', reverse, 5'-CCCAATACGACCAAATCCGTT-3'.

Immunohistochemical (IHC) analysis

Immunohistochemistry was done to examine UPKIa expression in 125 human colorectal carcinoma tissue specimens. UPKIa was detected using a goat anti-UPKIa polyclonal antibody (Santa Cruz, USA). Briefly, a paraffin section of the colorectal carcinoma tissue from the patient was deparaffinized with xylene and rehydrated. Antigenic retrieval was processed by submerging the sample in citrate buffer and microwaving. The sections were then treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity. Non-specific binding was blocked by treating the slides with 1% bovine serum albumin. The sections were then incubated overnight at 4C

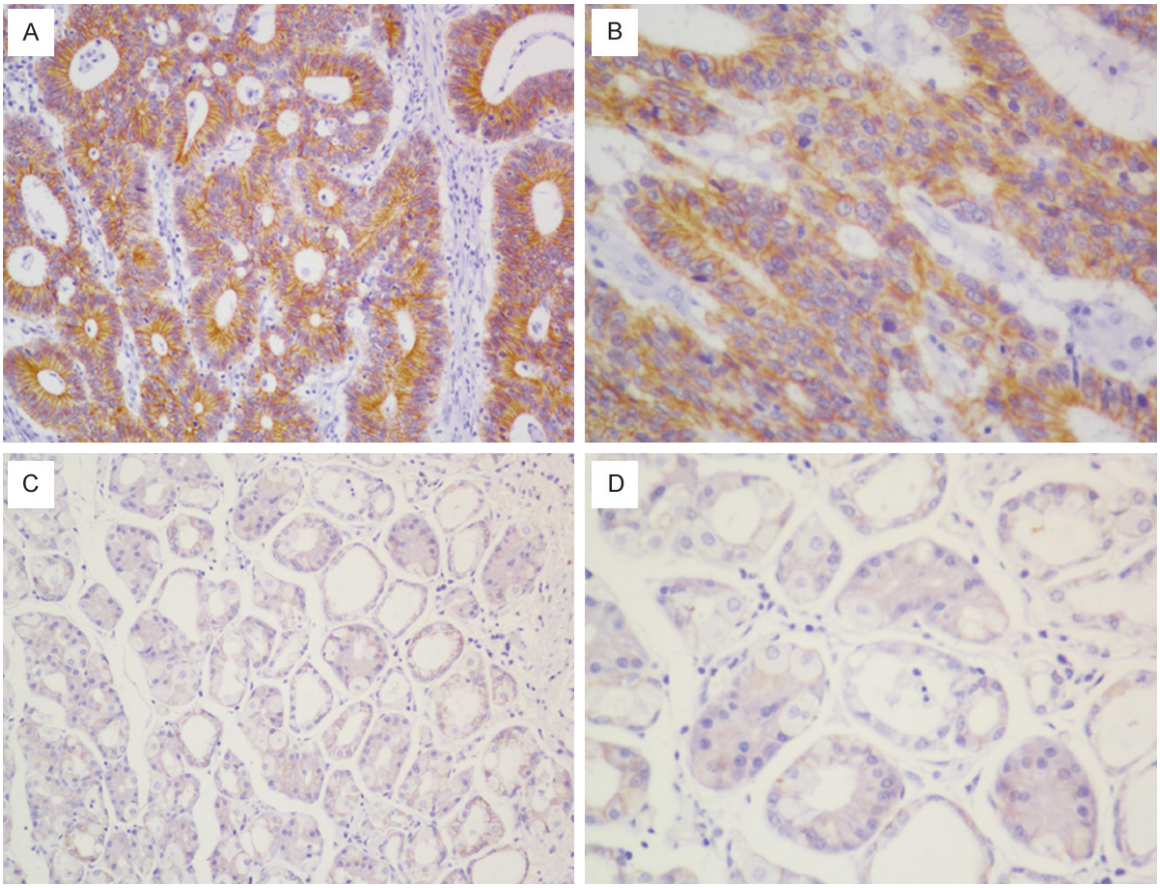

Figure 2. Strong (A, B) and weak (C, D) expression of UPKIa in colorectal cancer tissues by immunohistochemistry assays. (A, C with 200 x magnification; B, D with 400 x magnification).

with anti-UPKIa antibody (1:400). After washing, the tissue sections were then incubated with the biotinylated secondary antibody followed by further incubation with streptavidin-horseradish peroxidase complex. Finally, the sections were developed with diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin.

The degree of immunostaining of formalin-fixed, paraffin-embedded sections was reviewed and scored independently by two observers, based on both the proportion of positively stained tumor cells and the intensity of staining. The proportion of tumor cells was scored as follows: 0 (no positive tumor cells), 1 (< 10% positive tumor cells), 2 (10-50% positive tumor cells), 3 (50-80% positive tumor cells), and 4 (> 80% positive tumor cells). The intensity of staining was graded according to the following criteria: 0 (no staining); 1 (weak staining = light yellow), 2 (moderate staining = yellow brown), and 3 (strong staining = brown). The staining index (SI) was calculated as staining intensity score X proportion of positive tumor cells. Using this method of assessment, we evaluated the expression of AEG-1 in benign breast epithelium and malignant lesions by determining the SI, which scored as 0-4, 6, 9, and 12. Cutoff values for UPKIa were chosen on the basis of a measurement of heterogeneity with the log-rank test statistical analysis with respect to overall survival. An optimal cutoff value was identified: the SI score of ≥ 4 was used to define tumors as high UPKIa expression, and ≤ 3 as low expression of UPKIa.

Statistical analysis

All statistical analyses were carried out using the SPSS 17.0 statistical software package. The χ^2 test for proportion was used to analyze the relationship between UPKIa expression and clinicopathological characteristics. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. The sig-

Table 1. Correlation between UPKIa expression and clinicopathologic characteristics in colorectal cancer

Characteristics	UPKIA		*P*
	Low	High	
Age			0.804
< 60 y	41	29	
≥ 60 y	31	24	
Sex			0.729
Male	44	34	
Female	28	19	
UICC Stage			0.038
I-II	37	37	
III-IV	35	16	
Tumor size			0.035
≤ 5 cm	46	43	
> 5 cm	26	10	
Grade score			0.956
G1-2	56	41	
G3-4	16	12	
Preoperative CEA			0.326
≤ 5 ng/ml	39	24	
> 5 ng/ml	33	29	

nificance of various variables for survival was analyzed by the Cox proportional hazards model in the multivariate analysis. A $P < 0.05$ in all cases was considered statistically significant.

Results

Real-time PCR analysis

Real-time PCR was performed on 10 pairs of surgical specimens (colorectal cancer and adjacent non-tumor tissues) to examine the mRNA expression of UPKIa. There was a significant difference in the average expression level of UPKIa mRNA between the tumor tissues and the paired non-tumors (**Figure 1**), and the expression of UPKIa was lower in tumor tissues.

Immunohistochemistry analysis

To further investigate the expression of UPKIa in colorectal cancer, paraffin-embedded tissue (n = 125) were used for immunohistochemical analysis. The results showed that the positive UPKIa expression was localized to the membrane and cytoplasm (**Figure 2**). Seventy-three cases (58.4%) exhibited low expression of UPKIa (**Table 1**).

Association of UPKIa expression with clinicopathological characteristics

The correlation of UPKIa expression and clinicopathological factors were analyzed according to the IHC detection results. We observed that reduced UPKIa expression was significantly associated with UICC stage *(P* = 0.038) and tumor size (*P* = 0.035) (**Table 1**). Furthermore, we investigated the relationship of UPKIa expression and the clinical outcomes of the125 patients with colorectal cancer. The median observation period was 33.7 months (range, 0.5 to 60 months). The Kaplan-Meier survival analysis indicated that the OS (*P* = 0.017, **Figure 3A**) and RFS (*P* = 0.007, **Figure 3B**) were significantly higher in the higher UPKIa expression group compared with the lower UPKIa group. Multivariate Cox proportional hazards model analysis revealed that UPKIa expression was an independent prognostic factor for OS (*P* = 0.047, **Table 2**) and RFS (*P* = 0.029, **Table 2**).

Discussion

In this report, we present the first evidence that a tetraspanin protein, UPKIa, is downregulated in colorectal carcinoma tissues. UPKIa protein was observed in 92.7% of colorectal carcinoma specimens, and the expression level of UPKIa protein was found to be significantly associated with clinical staging and tumor size of colorectal tumor and the prognosis of patients with colorectal carcinoma.

Uroplakins are transmembrane N-glycosylated proteins originally found to be exposed at the luminal face of mammalian urothelium. UPIa and Ib contains a single N-glycosylation site in their large, extracellular loop that harbors high mannose and complex sugars, respectively. The prosequence portion of UPII contains three N-glycosylation sites, while the mature UPII does not have one. UPIII contains x sites that harbor complex sugars. Although such a glycosylation pattern is conserved among uroplakins of bovine, mouse and human, it can change as a function of urothelial differentiation as the glycosylation of uroplakins in cultured urothelial cells and transfected cells can vary [5, 11, 12]. UPKIa is a member of the transmembrane 4 superfamily, also known as the tetraspanin

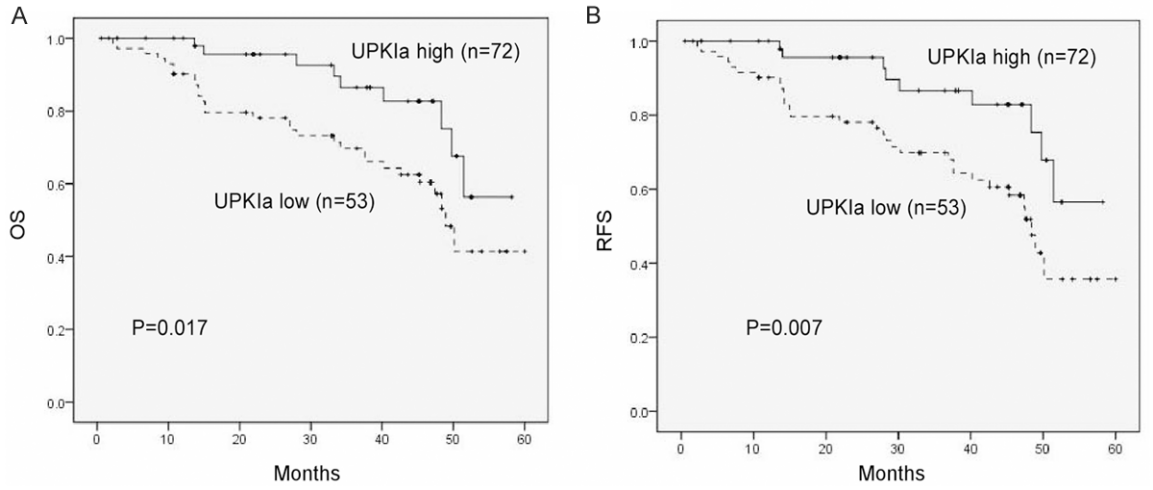

Figure 3. Kaplan-Meier curves of 125 patients inflicted with colorectal cancer with low expression versus high expression of UPKIa (A: OS; B: RFS).

Table 2. Multivariate analyses of various prognostic parameters in patients with colorectal cancer Cox-regression analysis

Prognostic variables	OS		RFS	
	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
Age (< 60 vs ≥ 60)	0.626 (0.081-4.854)	0.654	0.771 (0.147-5.774)	0.598
Sex (M vs F)	1.411 (0.409-2.119)	0.145	1.409 (0.265-2.146)	0.244
UICC Stage (III-IV vs I-II)	2.613 (0.704-9.696)	0.151	2.596 (0.493-6.213)	0.018
Grade score (G1-2 vs G3-4)	1.242 (0.394-3.914)	0.711	2.591 (0.694-9.677)	0.153
Tumor size (> vs ≤ 5 cm)	1.215 (0.496-2.987)	0.067	1.278 (0.545-2.998)	0.057
Preoperative CEA (> vs ≤ 5 ug/ml)	1.584 (0.448-5.139)	0.043	1.549 (0.472-5.087)	0.157
UPKIa expression (Low vs High)	2.632 (0.584-4.562)	0.047	2.729 (0.617-4.847)	0.029

family, which may play a role in the regulation of cell development, activation, growth and motility [13]. It has been shown that UPKIa may serve as the urothelial receptor for the FimH protein of uropathogenic Escherichia coli (UPEC), and that the binding of UPEC to UPKIa may play a crucial role in mediating the epithelial responses to UPEC attachment [14]. A correlation of UPKIa with cancer development and progression has recently been demonstrated by studies from several groups, in which the expression profiles of UPKIa vary in different types of human tumors. Kageyama et al. reported that high expression of UPKIa was observed in 94.4% moderately- to well-differentiated, while in 80.0% poorly differentiated urinary bladder transitional cell carcinoma [15]. Wang et al. found that the expression of UPKIa in gastric cancer was reduced in compared with adjacent non-tumor tissues [10]. Our study has provided evidence that UPKIa expression was downregulated in colorectal cancer tissues compared with normal colon tissues. Our finding further characterizes the expression patterns of UPKIa in human gastrointestinal tract cancers.

The expression of uroplakins has been related to the clinicopathologic features of several tumors. Wu et al. demonstrated that UPKII was might be a useful marker for detecting micrometastases of bladder cancer in the pelvic lymph nodes [16]. Huang et al. revealed that absent UPK expression was associated with advanced pathologic stage, lymph node status, recurrence, and cancer specific mortality in bladder cancer [17]. Matsumoto et al. reported that loss of UPKIII expression is associated with lymphovascular invasion, pathologic stage, grade, and decreased cancer-specific survival of aggressive bladder cancer [18]. It was not until recently that UPKIa was proposed to be

prognostically useful. Our study was in line with these studies in that downregulated expression of UPKIa was associated with the poor prognosis. Collectively, these studies described the prognostic roles of UPKIa in tumors from the same gastrointestinal tract organs. Further studies are still needed to evaluate the roles of UPKIa in predicting the prognosis in other tumors.

It is noteworthy that UPKIa has been found, in our study, to strongly correlate with clinical staging and tumor size. The best explanation may be that UPKIa, as a potential tumor suppressor gene, may be involved in tumor development and progression. This is supported by Cowled et al, who demonstrated that the promoter of UPKIb gene could be regulated by CpG methylation, in which Sp1 and NFκB, and other signaling and factors may be involved [19].

In conclusion, our findings suggest that the downregulation of UPKIa may be useful as a prognostic marker of colorectal cancer progression. Further study of the detailed molecular mechanism of UPKIa involvement in the development and progression of colorectal cancer is warranted. Nevertheless, our study has provided a basis for the development of a novel biomarker for the prognosis of colorectal cancer.

Disclosure of conflict of interest

None.

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