C5a receptor expression is associated with poor prognosis in urothelial cell carcinoma patients treated with radical cystectomy or nephroureterectomy

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Abstract. Patients with aggressive urothelial cell carcinoma (UCC) that undergo radical cystectomy or nephroureterectomy exhibit markedly high rates of disease recurrence and mortality. To select appropriate adjuvant thxerapies in addition to radical surgery, the identification of predictive prognostic markers for UCC patients is required. The aim of the present study was to identify such markers, by evaluating the association of UCC complement component 5 (C5) fragment a (C5a) receptor (C5aR) expression, detected using immunohistochemistry, with clinicopathological parameters and survival outcomes of UCC patients. The results revealed that C5aR was expressed in cancer cells, particularly at the invasive front, but not in noncancerous urothelial cells or adjacent cells. The UCC C5aR-positive rate of patients treated with radical surgeries was 73% (38/52) and the rate was 83% (20/24) at stages I-II of disease. No correlation between C5aR expression and any of clinicopathological parameters, which included gender, tumor location, World Health Organization grade, T stage, vessel invasion and stage of disease, was identified. However, univariate and multivariate analyses revealed that C5aR-positive UCC patients exhibited significantly lower overall survival rates [hazard ratio (HR), 3.14; 95% confidence interval (CI), 1.03-9.60; P=0.035 and HR, 3.92; 95% CI, 1.15-13.4; P=0.029, respectively] and 5-year survival rates (0.42 vs. 0.83) compared with C5aR-negative UCC patients. Furthermore, 5-year survival and disease-specific survival rates were lower in patients with C5aR-positive UCC (0.51; 95% CI, 0.30-0.71) than patients with C5aR-negative UCC (0.83; 95% CI, 0.62-1.00). These results indicate that UCC C5aR expression is predictive of poor patient outcomes and thus may lead to the appropriate selection of adjuvant therapies at earlier UCC stages, which could improve patient prognosis.

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Introduction

Urothelial cell carcinoma (UCC) is one of the most common types of cancer in the USA, accounting for ~4.5% of all newly diagnosed cancer cases and 2.8% of all cancer-associated mortalities in 2013 (1). In the USA, the most common site of UCC is the bladder, and bladder UCC is the fourth most common type of cancer and the sixth leading cause of cancer-associated mortality in males (1). Bladder UCC may be classified into two subtypes at diagnosis, non-muscle and muscle-invasive, which exhibit distinct clinical features (2). The non-muscle-invasive subtype commonly recurs in the bladder cavity and accounts for 70-80% of cases of bladder UCC, with muscle invasion present in only 10-20% of cases. The 5-year survival rate of the non-muscle-invasive subtype of UCC is >90%. By contrast, muscle-invasive bladder UCC, which accounts for ~20% of bladder UCC cases, exhibits a poor prognosis with a 5-year survival rate of <50%. Carcinoma *in situ* (CIS), which is classified into the non-muscle invasive aggressive subtype, is localized to the epithelium and exhibits invasive and metastatic potential. In patients with aggressive bladder UCC (muscle invasive UCC and CIS) without lymph nodes or distant metastasis, the standard and most effective treatment is total resection of the bladder and urethra, which includes the prostate in male patients. However, ≤50% of patients who undergo total cystectomy exhibit recurrence and develop distant metastases (3-5). Aggressive UCC with malignant potential may also originate from the upper urinary tract, renal calyx, renal pelvis and ureter (6). In patients with UCC of the upper urinary tract without lymph node or distant metastasis, the most effective treatment is total resection of the diseased section of the upper urinary tract, including the kidney, ureter and a section of the bladder (nephroureterectomy); however, ~40% of UCC patients that undergo total nephroureterectomy have succumbed to the disease 5 years after surgery (7). In general, patients with muscle invasive bladder UCC, CIS of

the lower urinary tract and UCC of the upper urinary tract are at high risk of recurrence, metastases and mortality even after radical surgery (8,9). Therefore, to improve prognosis of the patients with aggressive UCC, further treatments in addition to radical surgery are required. Presurgical neoadjuvant cisplatin chemotherapy with methotrexate, vinblastine, doxorubicin and cisplatin (10) and gemicitabin plus cisplatin (11) have been demonstrated to improve the survival of patients with muscle invasive UCC. Immunohistochemical markers are useful for the prediction of cancer behavior and appropriate treatment choice and thus may contribute to improving patient survival. The cell cycle regulators, p53, retinoblastoma protein, p21, p27 and cyclin E1, have been reported to predict disease recurrence and mortality following radical cystectomy in patients with pTa-pT3 node-negative UCC (12). A combination of these markers exhibited significantly higher predictive accuracy for disease recurrence and cancer specific mortality compared with isolated use of the markers; however, the use of individual markers did not improve the predictive accuracy (12-14). Therefore, the identification of a novel marker that predicts survival of patients with aggressive UCC more accurately is urgently required.

The anaphylatoxin complement component 5 fragment a (C5a) is an N-terminal 74-amino acid fragment that is released from the α-chain of C5, which functions as a leukocyte chemoattractant and inflammatory mediator (15,16). Increasing evidence has identified that the complement system is activated in human cancer tissues (17,18) and in animal cancer models (19,20), indicating that C5a may be present in the cancer microenvironment. C5a functions by binding to the C5a receptor (C5aR), which was originally identified in leukocyte cell lines (21). In our previous study, aberrant C5aR expression in cancer cells was identified in a number of different organs and C5a was demonstrated to enhance cancer cell invasiveness by activating motility and increasing matrix metalloproteinase release in a C5aR-dependent manner (22). Furthermore, direct C5a release from C5 by cell membrane proteases of cancer cells and promotion of C5aR-expressing cancer cell invasiveness by C5a was demonstrated, indicating that a self-activation circuit via C5aR exists in cancer cells that express C5aR and proteases on the cell surface (23). Notably, a previous study revealed that a C5aR agonist increased cancer cell proliferation and C5aR silencing reduced tumor growth (24). Thus, we hypothesize that C5aR-positive cancer cells are more aggressive than C5aR-negative cells and thus, C5aR expression in cancer cells may be associated with poor prognosis of cancer patients.

To determine whether C5aR expression affects the prognosis of patients with aggressive UCC undergoing radical surgery, the association of UCC C5aR expression with clinicopathological parameters and survival outcomes of patients was evaluated.

Materials and methods

Patients and UCC samples. Cancerous and non-cancerous adjacent tissues were obtained from patients during surgery. A total of 52 patients with aggressive UCC who underwent radical cystectomy for bladder UCC (41 patients; 78%) or radical nephroureterectomy for upper urinary tract UCC (11 patients; 22%) at Kumamoto University Hospital (Kumamoto, Japan) between April 1996 and March 2013 were enrolled in the present study (Table I). The patient cohort included 39 male patients and 13 female patients with a median age of 72 years (range, 43-86 years). The median duration of follow-up was 27.4 months (range, 0.53-150.0 months). A total of 16 patients (30.8%) succumbed to urothelial cancer and 5 patients (9.6%) succumbed due to other causes (4 pneumonitis; 1 unknown). Written informed consent for the use cancer and adjacent noncancerous tissues and clinicopathological records was obtained from all the patients prior to surgery. The Kumamoto University Hospital Ethics Committee approved the use of the samples and records for the present study.

Immunohistochemistry and retrospective analysis. Deparaffinized tissue sections (2-μm) were pretreated with 0.3% H_2O_2 in methanol for 20 min, followed by treatment with Protein Block Serum-Free (Dako Cytomation, Carpinteria, CA, USA) for 20 min. Sections were incubated with primary mouse anti-human C5aR antibody (2 μg/ml; dilution, 1:50; catalog no., HM2094; Hycult Biotech, Uden, The Netherlands) or nonspecific mouse IgG (2 μg/ml; dilution, 1:50; catalog no., X0944; Dako Cytomation) at room temperature for 1 h followed by staining with EnVision+ solution (Dako Cytomation) and 3,3'-diaminobenzidine tetrahydrochloride solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 0.006% H_2O_2, according to the manufacturer's instructions. Nuclei were counterstained with hematoxylin (Kanto Chemical Co., Inc., Tokyo, Japan) and visualized with an optical microscope. Histopathological analyses were performed by senior pathologists and reviewed by the chief of clinical pathology. All tumors used in this study were classified as UCC according to the World Health Organization (WHO) grading criteria (25,26). The association between UCC C5aR expression and patient survival, tumor stage and grade was analyzed. All the analyses were conducted by investigators blinded to the outcome of the patients.

Statistical analysis. In the analysis of clinicopathological parameters in association with UCC C5aR expression, Fisher's exact test was used, with the exception of age, which was analyzed with the Mann-Whitney U test. Kaplan-Meier analysis was performed to calculate 5-year survival rates and the log-rank test was conducted to analyze overall survival (OS). The Cox proportional hazards model was used to calculate the hazard ratio (HR) and 95% confidence interval (CI) for univariate and multivariate analysis. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS version 20.0 statistical software (IBM Corporation, Armonk, NY, USA).

Results

C5aR expression in UCC. C5aR expression was identified in 38 UCC patients (73%) (Table I). C5aR staining (Fig. 1) was localized to the UCC cell membrane (Fig. 1A-C), however, no C5aR expression was identified in noncancerous urothelial cells or adjacent cells (Fig. 1F). Notably, UCC cells at the invasive front rather than the surface layer expressed C5aR, and almost all UCC cells in the deeper invasion site were C5aR-positive (Fig. 1D and E).

Table I. Association between C5aR expression and the clinicopathological parameters of 52 urothelial carcinoma cancer patients.

Parameters	Patients, n[a]	C5aR (+), n	C5aR (-), n	P-value
Age				
Median, years (range)	52	73.0 (43-86)	66.5 (53-83)	0.3640[b]
Gender				
Male	39	28	11	0.5116[c]
Female	13	10	3	
Tumor location				
Upper urinary tract	11	7	4	0.3302[c]
Bladder	41	31	10	
WHO grade				
G1-2	13	10	3	0.5047[c]
G3	35	25	10	
T stage				
T1-2	28	22	6	0.3659[c]
T3-4	20	14	6	
Blood vessel invasion, +/-	19/26	13/21	6/5	0.2726[c]
Lymph node invasion, +/-	19/28	13/22	6/6	0.3265[c]
Stage of disease				
I-II	24	20	4	0.1590[c]
III-IV	24	16	8	

[a]Where the total number of patients does not equal 52, information concerning these parameters could not be obtained from certain patients. [b]P-value determined using the Mann-Whitney U test. [c]P-value determined using Fisher's exact test. (+), positive expression; (-), negative expression; C5aR, complement component 5 fragment a receptor; WHO, World Health Organization.

Table II. Univariate and multivariate analysis of overall survival in 52 urothelial carcinoma patients.

Parameter	5-year survival rate	Univariate analysis			Multivariate analysis		
		HR	95% CI	P-value	HR	95% CI	P-value
Location (PU/B)	0.62/0.53	1.89	0.56-6.45	0.299	2.02	0.51-7.94	0.313
C5aR expression (-/+)	0.83/0.42	3.14	1.03-9.60	0.035	3.92	1.15-13.4	0.029
WHO grade (3/1-2)	0.54/0.50	1.04	0.38-2.88	0.938	1.46	0.48-4.41	0.508
Stage of disease (I-II/III-IV)	0.64/0.43	1.19	0.77-4.82	0.156	2.30	0.86-6.16	0.099

Kaplan-Meier analysis method was used to calculate the 5-year survival rate. Cox proportional hazards regression was used to calculate the HR and 95% CI. PU/B, pelvic vs. ureter/bladder; -/+, negative vs. positive; 3/1-2, 3 vs. 1 and 2; 1-2/3-4; I and II vs. III and IV; HR, hazard ratio; CI, confidence interval; WHO, World Health Organization.

Association between UCC C5aR expression and clinicopathological parameters. The associations between clinicopathological parameters and C5aR expression were investigated (Table I). Previously, age was demonstrated as a risk factor for developing UCC of the bladder and advanced bladder UCC is more common in females who exhibit worse survival rates than males (27). Since there was no significant association of UCC C5aR expression with age and gender in the present study, the two parameters are unlikely to show bias towards the low overall survival rate of patients with C5aR-positive UCC. No significant differences in C5aR expression were identified between different UCC locations, including the bladder and upper urinary tract. Furthermore, UCC C5aR expression was not associated with tumor grade, pathological tumor stage, vessel invasion or clinical stage; however, it is notable that UCC C5aR-positive rate was markedly high in tumors at T1-2 and of patients at stage I-II.

Association between UCC C5aR expression and patient survival. To determine whether C5aR expression correlated with UCC patient prognosis, patient survival times were investigated. No significant differences in 5-year survival rate and OS rate were identified between UCC of the upper (pelvic/ureter) and lower (bladder) urinary tract (Table II),

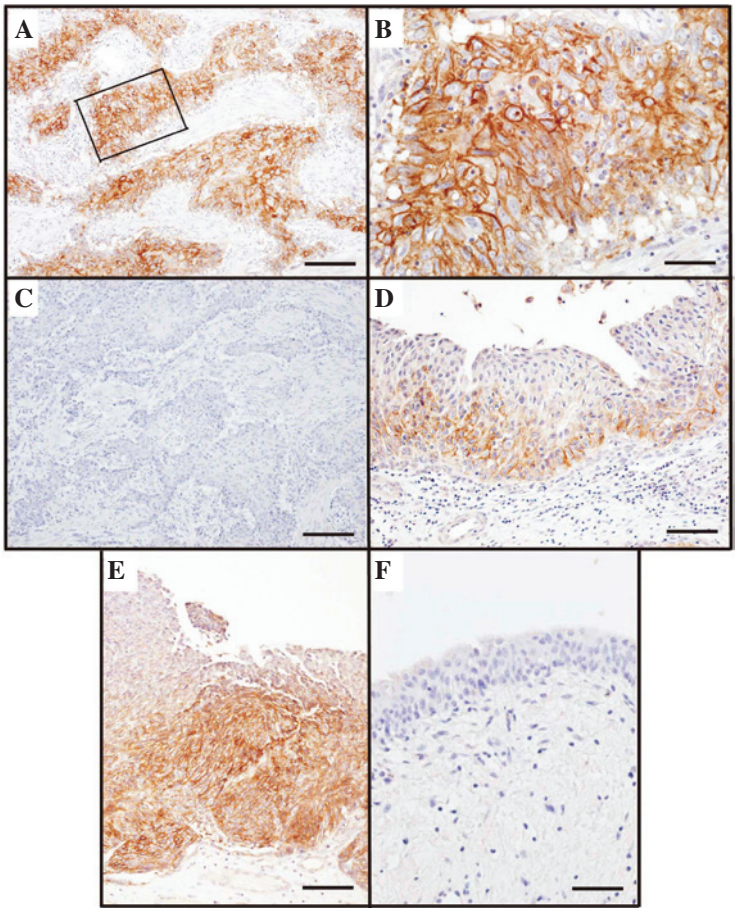

Figure 1. Immunohistochemical staining of UCC tissues for C5aR. Tissue sections were immunostained with anti-human C5aR IgG antibodies. (A) UCC tissue in the deep invasion site; scale bar, 200μm. (B) Magnified UCC tissue surrounded by the square in part A; scale bar, 50μm. (C) Same UCC tissue stained with nonspecific IgG; scale bar, 200μm. (D) UCC localized in the surface area; scale bar, 100μm. (E) UCC of advanced invasion; scale bar, 100μm. (F) Noncancerous urothelial cell layer; scale bar, 50μm. UCC, urothelial cell carcinoma; C5aR, C5a receptor; IgG, immunoglobulin G.

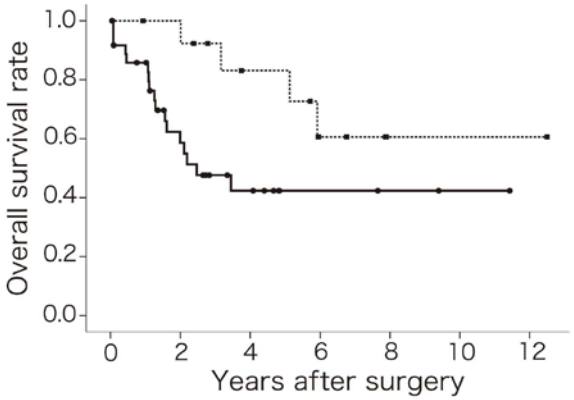

Figure 2. Overall survival rates of patients with C5aR-positive (solid line) and C5aR-negative (dashed line) UCC. C5aR, complement component 5 fragment a receptor; UCC, urothelial cell carcinoma.

which enabled survival rate analysis to be conducted for all patients in this study. The OS rate of C5aR-positive UCC patients was lower than that of C5aR-negative patients (Fig. 2). Furthermore, the 5-year survival rate of C5aR-positive UCC patients was ~50% lower compared with C5aR-negative UCC patients (Table II). Univariate (HR, 3.14; P=0.035) and multivariate (HR, 3.92; P=0.029) analyses revealed that the OS rate of patients with C5aR-positive UCC was significantly lower than that of patients with C5aR-negative UCC. However, no significant differences in OS rate were identified between UCC WHO grade or stage of disease (Table II). Furthermore, UCC C5aR expression was not associated with the disease-specific survival rate of patients (P=0.092); however, the disease-specific 5-year survival rate was significantly lower in patients with C5aR-positive UCC (0.51; 95% CI, 0.30-0.71) than patients with C5aR-negative UCC (0.83; 95% CI, 0.62-1.00) (P=0.032).

Discussion

The present study identified that C5aR is expressed in aggressive type UCC cells and revealed that patients with C5aR-positive UCC exhibit lower survival rates when compared with C5aR-negative UCC patients. Aggressive type UCC cells expressed C5aR at the greatest rate (73%) (Fig. 1; Table I) compared with the C5aR-positive rates of other types of cancer, as previously reported (22). This finding may indicate an association between C5aR expression and UCC aggressiveness. Invasiveness of C5aR-expressing cancer cells is augmented by C5a (22), which is present in the cancer microenvironment (17-20). UCC cells at the invasive front are in direct contact with interstitial fluid that contains C5, which

facilitates C5a release by cancer cell proteases (23). C5aR expression in UCC cells at the invasive front (Fig. 1D and E) appears to be correlated with the invasiveness of aggressive UCC (3). Thus, the poor outcomes of patients with C5aR-positive UCC (Fig. 2; Table II) may be associated with the invasive potential of C5a-activated C5aR-positive cancer cells (22).

Pathological factors have been widely used to predict the outcomes of patients with cancer, including UCC. In UCC, advanced tumor stage and grade are associated with distant metastasis and increased invasive potential (28). In addition, tumor size (diameter ≥3 cm) and lymph node invasion indicate a worse prognosis for UCC patients (29,30). However, ~20% of aggressive UCC patients that undergo total cystectomy experience recurrence (4) and the survival rate of UCC patients that undergo total nephroureterectomy is relatively low (~60%) (7). These studies indicate the limited efficacy of radical surgeries and highlight the urgent requirement for other reliable prognostic/predictive markers to identify patients that require additional treatments to decrease the risk of cancer recurrence and mortality. In the present study, C5aR expression was identified as a possible negative prognostic factor for aggressive UCC (Table II). In this study, C5aR-expressing UCC patients exhibited the shortest OS times and the highest HRs, which may indicate that disease-associated mortality is more closely correlated with C5aR expression than high cancer grade or advanced clinical stage. In addition, no significant associations were identified between UCC C5aR expression and negative prognostic factors, including tumor grade and stage, vessel invasion or clinical stage (Table I). Multivariate analysis revealed that C5aR-positive UCC patients exhibited a significantly lower OS rate than C5aR-negative UCC patients (Table II). Therefore, these results indicate that C5aR expression is an independent prognostic factor of poor outcomes for UCC patients.

A previous study demonstrated a synergistic effect of the apoptosis markers, Bcl-2, caspase-3, p53 and survivin, on the progression of bladder cancer and revealed that changes in the expression of the four markers in patients that underwent radical cystectomy was independently predictive of high risk for disease recurrence and mortality (31). Although it has not yet been demonstrated in human UCC, C5aR signaling suppresses apoptosis, induces T-cell expansion via the enhanced expression of Bcl-2 (32) and enhances neuronal cell survival via inhibition of caspase-3 activation (33). C5a is present in the cancer microenvironment (17,20,23), where, by stimulation with C5a, the apoptosis of C5aR-expressing UCC may be suppressed; this supression facilitates the growth and development of cancer in concert with the C5a-elicited enhancement of proliferation (24) and invasiveness (22). Thus, C5aR expression in UCC may correlate with the low survival rate exhibited by patients (Table II). It has been reported that high C5aR mRNA expression in cancer cells is associated with short survival time in patients with ovarian or lung cancer (24), in accordance with the results of the present study, which revealed that C5aR protein expression of UCC cells correlated with low survival rates in UCC patients (Fig. 2; Table II). These results indicate that cancer cell C5aR expression is a marker of poor prognosis.

In bladder cancer patients that underwent radical cystectomy, survivin overexpression was demonstrated to be associated with increased overall mortality, cancer specific mortality and tumor recurrence (34). However, increased survivin expression was also associated with advanced pathological stage, lymphovascular invasion and lymph node metastasis, which indicates that survivin expression is lower at the early stages of UCC than the advanced stages. By contrast, higher C5aR expression at organ-confined cancer stages, T1 and T2, and higher HR of C5aR expression as an independent prognostic factor of poor outcomes (Tables I and II), indicates that UCC C5aR expression may lead to the use of additional treatments at relatively early stages of disease for patients undergoing surgery. Since C5aR-positive UCC patients exhibit a lower disease-specific 5-year survival rate than C5aR-negative UCC patients, additional treatments at the earlier stages of disease may improve prognosis. Further study is required to establish an association between UCC C5aR expression and clinical outcomes, including cancer recurrence, progression and mortality, for early stage UCC patients with C5aR-positive cells in the urine or tissues obtained from transurethral resection or bladder tumor biopsy.

In conclusion, UCC C5aR expression was identified as an independent marker of poor prognosis in aggressive UCC in the present study. Notably, C5aR expression is higher at earlier stages of UCC and thus, immunohistochemical staining of C5aR in UCC may be applicable for clinical examination (cytological analysis of urine and/or biopsied tissues), to provide information regarding tumor aggressiveness. This information may also be used to predict the prognosis of UCC patients. Future studies that evaluate the efficacy of additional treatments, such as adjuvant chemotherapy, radiotherapy and/or molecular-targeted therapy, for the prevention of recurrence and improvement of survival outcomes in C5aR-positive UCC patients are required.

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