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Urologic Oncology: Seminars and Original Investigations 37 (2019) 293.e11−293.e24

UROLOGIC ONCOLOGY

Original Article

High TNFAIP6 level is associated with poor prognosis of urothelial carcinomas

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Received 14 October 2018; received in revised form 3 December 2018; accepted 8 December 2018

Abstract

Purpose: Inflammatory responses affect each stage of carcinogenesis, from initiation, through invasion, to metastasis. Studies have shown that chronic inflammation induced by environmental and occupational exposures increase the risk of developing urothelial carcinoma (UC). Using a published UC transcriptome (GSE32894), we identified that among genes associated with inflammatory response (GO:0006954), TNFAIP6 was significantly upregulated during UC progression. Therefore, we investigated the association of TNFAIP6 with disease features, metastasis and survival in our well-characterized cohort of UC.

Methods: We determined TNFAIP6 expression in 340 upper urinary tract UCs (UTUC) and 295 urinary bladder UCs (UBUC) using immunohistochemistry and evaluated the results using H-score. TNFAIP6 expression correlated with clinicopathological features, disease-specific survival, and metastasis-free survival. Survival analysis was performed using Kaplan−Meier curves and Cox proportional hazards model.

Results: High TNFAIP6 expression was significantly associated with advanced pathological stage, lymph node metastasis, perineural invasion, vascular invasion, and high mitotic activity. Multivariate analysis identified high TNFAIP6 expression as an independent predictor of disease-specific survival (hazard ratio in UTUC: 2.891, $P = 0.003$; in UBUC: 2.175, $P = 0.017$) and metastasis-free survival (hazard ratio in UTUC: 3.803, $P < 0.001$; in UBUC: 3.845, $P < 0.001$).

Conclusion: High TNFAIP6 expression is associated with aggressive clinicopathological features and poor prognosis in UCs, suggesting it may serve as a novel prognosticator and treatment target. TNFAIP6 immunostaining may be used with current pathological examinations for better risk stratification for UCs.

Keywords: Urothelial carcinoma; *TNFAIP6*; Immunostaining; Transcriptome; Prognosis

Funding: This study was supported by Taiwan Ministry of Science and Technology (MOST106-2314-B-037 -058, MOST 107-2314-B-037-013), Kaohsiung Medical University Hospital (KMUH103-3T06, KMUH104-4T03, KMUH105-5R48), and the Biobank at Chi Mei Medical Center. All funders had no role in study design, data collection and analysis, interpretation of results, decision to publish, or preparation of the manuscript.

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https://doi.org/10.1016/j.urolonc.2018.12.009
1078-1439/

1. Introduction

Urothelial carcinoma (UC) is a major malignancy of the genitourinary tract that commonly occurs in the urinary bladder (UB) [1−4]. Upper urinary tract UC (UTUC) is rare in western countries but is prevalent in Taiwan [5−7]. Many important risk factors associated with chronic inflammation contribute to UC development and progression, including tobacco smoking, exposure to aristolochic acid, occupational exposure to aromatic amines, chlorinated hydrocarbons, and polycyclic aromatic hydrocarbons [1−10].

Radical surgery is the only curative treatment for UC [2−6]. The main prognosticators of UC are pathological stage, grade, lymphovascular invasion, and lymph node metastasis [2−6]. However, tumor recurrence and metastasis usually develop in the future, resulting in poor prognosis. Despite recent advances in surgical techniques and combined anti-UC modalities, the mortality rate associated with UC has not significantly decreased in the past 2 decades [1−6]. Therefore, better biological understanding of UC biology is imperative for improving patient treatment and prognosis.

The inflammatory microenvironment plays an important role in tumorigenesis, and is involved in tumor initiation, promotion, malignant transformation, invasion, and metastasis [11,12]. Inflammation also affects immune surveillance and responses to therapy [11,12]. In UC, inflammatory response and the presence of inflammatory cells, such as regulatory T cells, tumor-associated macrophages, and dendritic cells, and cytokines, for instance tumor necrosis factor alpha (TNFα) and interleukins contribute to the development of the inflammatory microenvironment, which is associated with UC formation and progression [8,13,14]. However, the molecular mechanisms underlying this process remains largely unknown. For identifying the important markers of UC-associated inflammation related to UC, we carried out data mining and processing, focusing on inflammatory response, using a public gene expression profiling dataset available in Gene Expression Omnibus (GEO). We observed that high expression level of TNFα induced protein 6 (*TNFAIP6*) correlated significantly with advanced pathological stage in UBUCs, signifying its role in cancer progression.

TNFAIP6 also named as TNF-stimulated gene 6 protein is a member of the hyaluronan-binding family of proteins, and plays an important role in the protease network associated with inflammation. TNFAIP6 is also involved in extracellular matrix (ECM) remodeling, cell adhesion, and cell migration. TNFAIP6 has been well-studied with respect to inflammatory disease, but its role in cancer is still being investigated [15,16]. Patients with colon and ovary cancer and high TNFAIP6 expression are predicted to have worse prognostic outcomes [17,18]. However, the possible prognostic roles of TNFAIP6 in UC remain unclear. Hence, we intended to comprehensively analyze the association of TNFAIP6 expression between clinicopathological factors and its impact on patients' outcomes in our characterized UC cohort.

2. Materials and methods

2.1. Data mining using the GEO database

We performed data mining on 1 dataset (GSE32894) of the GEO (National Center for Biotechnology information, Bethesda, MD) database derived from 308 UBUC radical specimens and analyzed it using HumanHT-12 V3.0 Expression BeadChip (Illumina, Inc., San Diego, CA). To computerize the expression level, we imported the raw files into the Nexus Expression 3 software (BioDiscovery, El Segundo, CA) and analyzed all probe sets without filtering or preselection. Under supervision, we conducted a comparative analysis to assess statistically significant gene expression changes in tumor progression. We compared the different transcriptomic levels between high-stage (T2−T4) and low-stage (Ta−T1) UBUCs to identify functional profiles, focusing on genes related to inflammatory response (GO:0006954). Only genes showing significantly different expression (log2 ratio >0.1, $P < 0.01$) were selected for further validation.

2.2. Patients and tumor specimens

We retrieved available formalin-fixed, paraffin-embedded tissue blocks from 295 UBUC and 340 UTUC patients between January 1998 and December 2004. All patients underwent surgical treatment with curative intent and none received preoperative radiotherapy or chemotherapy. We retrospectively reviewed the medical charts for each patient and collected clinical and pathological data. The pathological grade was classified according to 1998 WHO histological criteria, and tumor stage was determined in accordance with the 2002 tumor node metastasis classification. The institutional review board of Chi Mei Medical Center approved this study (IRB10501-005).

2.3. TNFAIP6 immunohistochemical staining and scoring

As described previously [19], paraffin tissue blocks were sectioned into 4-μm-thickslices and placed on precoated slides. We followed the standard procedures, including deparaffinization, rehydration, and antigen retrieval. Next, endogenous peroxidase was blocked by 3% H_2O_2 treatment. The slides were then incubated with primary antibody against TNFAIP6 (1:50, LS-C177123, LifeSpan BioSciences Inc) for 1 hour, followed by antibody detection using a ChemMate EnVision kit (K5001; DAKO, Glostrup, Denmark). Sections incubated without the primary antibody were used as negative controls. Two independent pathologists evaluated the tumor cells intensity and distribution of immunostaining to generate the H-score using the formula $\Sigma Pi(i+1)$, where i is the intensity of stained tumor cells (0−3+), and Pi is the percentage of

stained tumor cells (0%–100%) [20]. Based on the median H-score, the immunoreactivity was bisected into high and low expression levels.

2.4. Statistical analysis

All calculations were conducted using the SPSS software, version 17.0 (IBM, Armonk, NY). We used Pearson's chi-square test to assess the association of various clinicopathological parameters and TNFAIP6 expression. Two end points, disease-specific survival (DSS), and metastasis-free survival (MFS) were analyzed. Survival rates were calculated from the date of curative surgery to the date of an event or last visit. With regard to DSS or MFS the predictive value of the clinical and pathological parameters was analyzed. Univariate analysis was performed using the Kaplan–Meier curves with log-rank test. To identify the independent predictors, we included all significant factors for univariate analysis in the multivariate Cox proportional hazards model. For all analyses statistical significance was assessed at $P < 0.05$.

3. Results

3.1. Data mining confirmed that *TNFAIP6* transcript was significantly upregulated in UBUC

We recognized 37 probes covering 32 transcripts associated with inflammatory response (GO:0006954) from the published transcriptomic profiles of the GSE32894 dataset. These genes were significantly upregulated with increment in pT status (Fig. 1 and Table 1). We selected *TNFAIP6* from among these genes, as *TNFAIP6* is not only an important regulator of inflammatory response, but is also involved in ECM remodeling and cell migration, which are key processes of tumor progression. These observations prompted us to further study the role of TNFAIP6 in our large UC cohort.

3.2. Clinicopathological characteristics of UCs

We analyzed data of 635 UC patients, including 340 patients with UTUCs and 295 with UBUCs, with mean age of 65.8 ± 11.0 years (range: 25–91 years) (Table 2). The UTUC cohort contained 62 patients (18.2%) with multiple tumors, and 49 (14.4%) had concurrent renal pelvic and ureteral tumors. Two hundred and eighty-four patients (83.5%) harbored high histological grade tumors. Advanced UTUC (pT2–T4) was noted in 159 cases (46.8%), and167 lesions (49.1%) showed high mitosis. Perineural invasion (PNI) and vascular invasion (VI) were noted in 19 (5.9%) and 106 cases (31.2%), respectively. Metastatic lymph node was identified in 28 patients (8.2%). In the UBUC cohort, majority of cases ($n = 239$, 81%) were of high histological grade; 123 patients (41.7%) had advanced UBUC (pT2–T4) at initial diagnosis and 156 tumors (52.9%) showed high mitotic activity. Regarding lymph node status, 29 patients (7.8%) harbored N1-2 and 266 (92.2%) had N0 tumors. In addition, PNI and VI were observed in 49 (16.6%) and 20 tumors (6.8%), respectively.

3.3. Association between TNFAIP6 expression and clinicopathological features

Immunohistochemical staining showed variable TNFAIP6 expression, which was elevated in high-stage UC and weak in low-stage UC (Fig. 2). We then correlated TNFAIP6 expression with the clinical and pathological characteristics (Table 2).

In UTUC, TNFAIP6 expression level was significantly high in the advanced stage ($P < 0.001$), PNI ($P < 0.001$), VI ($P = 0.002$), and lymph node metastasis ($P = 0.049$). In UBUC, high TNFAIP6 expression significantly correlated with advanced stage ($P < 0.001$), VI ($P = 0.045$), and high mitotsis ($P = 0.042$).

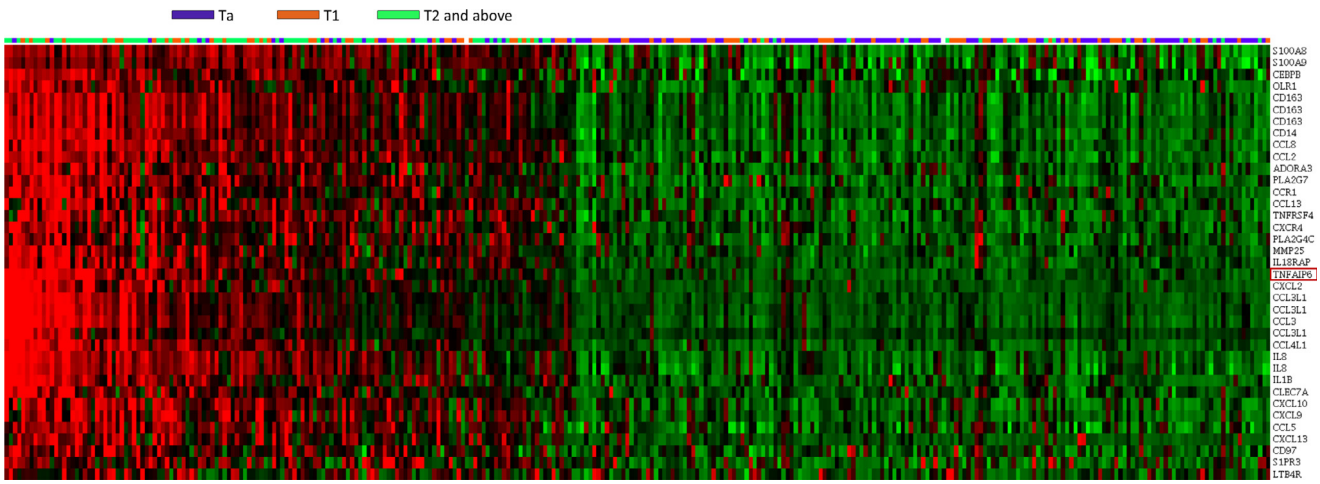

Fig. 1. Expression profiles of genes associated with inflammatory response (GO:0006954) extracted from a published UBUC transcriptome (GSE32894) in Gene Expression Omnibus. The expression levels of 37 probes targeting 32 genes were identified as significantly different (log2 ratio >0.1, $P < 0.01$) in high-stage (pT2–T4) tumors compared to low-stage (pT0–T1) tumors. Abbreviations: UBUC=urinary bladder urothelial carcinoma.

Table 1
Summary of differentially expressed genes associated with inflammatory response in the transcriptome of urothelial carcinoma of urinary bladder (GSE32894).

Probe	Comparing T1−Ta		Comparing T2-4−T1		Comparing T2-4−Ta		Gene symbol	Biological process	Molecular function
	Log ratio	*P* value	Log ratio	*P* value	Log ratio	*P* value			
ILMN_1729801	1.199	0.0001	1.375	<0.0001	2.574	<0.0001	*S100A8*	Inflammatory response	Calcium ion binding, protein binding
ILMN_1666733	0.7106	0.0032	1.281	<0.0001	1.9916	<0.0001	*IL8*	G-protein coupled receptor protein signaling pathway, angiogenesis, calcium-mediated signaling, cell cycle arrest, cell motion, cell-cell signaling, chemotaxis, immune response, induction of positive chemotaxis, inflammatory response, intracellular signaling cascade, negative regulation of cell proliferation, neutrophil activation, neutrophil chemotaxis, regulation of cell adhesion, regulation of retroviral genome replication	Chemokine activity, interleukin-8 receptor binding, protein binding
ILMN_2184373	0.7671	0.0025	1.201	<0.0001	1.9681	<0.0001	*IL8*	G-protein coupled receptor protein signaling pathway, angiogenesis, calcium-mediated signaling, cell cycle arrest, cell motion, cell-cell signaling, chemotaxis, immune response, induction of positive chemotaxis, inflammatory response, intracellular signaling cascade, negative regulation of cell proliferation, neutrophil activation, neutrophil chemotaxis, regulation of cell adhesion, regulation of retroviral genome replication	Chemokine activity, interleukin-8 receptor binding, protein binding
ILMN_1750974	1.1322	0.0001	0.7604	<0.0001	1.8926	<0.0001	*S100A9*	Actin cytoskeleton reorganization, cell-cell signaling, inflammatory response, leukocyte chemotaxis, regulation of integrin biosynthetic process	Calcium ion binding, protein binding, signal transducer activity
ILMN_1791759	0.9236	<0.0001	0.8283	<0.0001	1.7519	<0.0001	*CXCL10*	Blood circulation, cell surface receptor linked signal transduction, cell-cell signaling, chemotaxis, immune response, inflammatory response, muscle organ development, positive regulation of cell proliferation	cAMP-dependent protein kinase regulator activity, chemokine activity
ILMN_1772964	0.8592	<0.0001	0.8462	<0.0001	1.7055	<0.0001	*CCL8*	Calcium ion transport, cell-cell signaling, chemotaxis, exocytosis, immune response, inflammatory response, response to virus, signal transduction	Chemokine activity, heparin binding, signal transducer activity
ILMN_1720048	0.7005	<0.0001	0.783	<0.0001	1.4836	<0.0001	*CCL2*	G-protein signaling; coupled to cyclic nucleotide second messenger, JAK-STAT cascade, anti-apoptosis, cell adhesion, cellular calcium ion homeostasis, chemotaxis, cytokine-mediated signaling pathway, humoral immune response, inflammatory response, macrophage chemotaxis, monocyte chemotaxis, organ morphogenesis, protein amino acid phosphorylation, viral genome replication	G-protein-coupled receptor binding, chemokine activity, protein kinase activity, signal transducer activity
ILMN_1745356	0.7493	<0.0001	0.7062	<0.0001	1.4555	<0.0001	*CXCL9*	G-protein coupled receptor protein signaling pathway, cell-cell signaling, cellular defense response, chemotaxis, immune response, inflammatory response, signal transduction	Chemokine activity

(*continued on next page*)

Table 1 (***Continued***)

Probe	Comparing T1−Ta		Comparing T2-4−T1		Comparing T2-4−Ta		Gene symbol	Biological process	Molecular function
	Log ratio	*P* value	Log ratio	*P* value	Log ratio	*P* value			
ILMN_2379599	0.5633	<0.0001	0.8675	<0.0001	1.4307	<0.0001	*CD163*	Acute-phase response, inflammatory response	Protein binding, scavenger receptor activity
ILMN_2100209	0.5519	0.0001	0.7725	<0.0001	1.3244	<0.0001	*CCL4L1*	Chemotaxis, immune response, inflammatory response	Chemokine activity
ILMN_1722622	0.5065	<0.0001	0.8138	<0.0001	1.3203	<0.0001	*CD163*	Acute-phase response, inflammatory response	Protein binding, scavenger receptor activity
ILMN_1740015	0.4017	0.001	0.8874	<0.0001	1.2891	<0.0001	*CD14*	Apoptosis, cell surface receptor linked signal transduction, immune response, inflammatory response, phagocytosis, response to molecule of bacterial origin	Opsonin receptor activity, peptidoglycan receptor activity, protein binding
ILMN_1785732	**0.2886**	**0.0076**	**0.9937**	**<0.0001**	**1.2823**	**<0.0001**	***TNFAIP6***	Cell adhesion, cell-cell signaling, inflammatory response, signal transduction	Hyaluronic acid binding, protein binding
ILMN_1671509	0.5535	<0.0001	0.6831	<0.0001	1.2366	<0.0001	*CCL3*	G-protein coupled receptor protein signaling pathway, cell motion, cell-cell signaling, cellular calcium ion homeostasis, chemotaxis, cytoskeleton organization, exocytosis, immune response, inflammatory response, regulation of viral genome replication, signal transduction	Chemoattractant activity, chemokine activity, signal transducer activity
ILMN_1775501	0.4625	0.0066	0.7662	<0.0001	1.2287	<0.0001	*IL1B*	Activation of MAPK activity, anti-apoptosis, apoptosis, cell-cell signaling, cytokine-mediated signaling pathway, fever, immune response, inflammatory response, leukocyte migration, negative regulation of cell proliferation, neutrophil chemotaxis, positive regulation of I-kappaB kinase/NF-kappaB cascade, positive regulation of JNK cascade, positive regulation of chemokine biosynthetic process, positive regulation of interleukin-6 biosynthetic process, positive regulation of interleukin-6 production, positive regulation of nitric oxide biosynthetic process, positive regulation of protein amino acid phosphorylation, positive regulation of transcription factor activity, regulation of insulin secretion, signal transduction	Interleukin-1 receptor binding, protein binding, signal transducer activity
ILMN_1701195	0.6995	<0.0001	0.517	<0.0001	1.2165	<0.0001	*PLA2G7*	Inflammatory response, lipid catabolic process	1-alkyl-2-acetylglycerophosphocholine esterase activity, hydrolase activity, phospholipid binding
ILMN_1723035	0.4317	0.0019	0.6657	<0.0001	1.0974	<0.0001	*OLR1*	Blood circulation, cell adhesion, immune response, inflammatory response, proteolysis	Low-density lipoprotein receptor activity, protein binding, receptor activity, sugar binding
ILMN_1733270	0.3801	<0.0001	0.7058	<0.0001	1.086	<0.0001	*CD163*	Acute-phase response, inflammatory response	Protein binding, scavenger receptor activity
ILMN_2098126	0.513	0.0062	0.5293	0.0011	1.0423	<0.0001	*CCL5*	Cell adhesion, cell motion, cell-cell signaling, cellular calcium ion homeostasis, cellular defense response,	

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Table 1 (***Continued***)

Probe	Comparing T1−Ta		Comparing T2-4−T1		Comparing T2-4−Ta		Gene symbol	Biological process	Molecular function
	Log ratio	*P* value	Log ratio	*P* value	Log ratio	*P* value			
								chemotaxis, exocytosis, immune response, inflammatory response, negative regulation of viral genome replication, response to oxidative stress, response to virus, signal transduction	Chemoattractant activity, chemokine activity, signal transducer activity
ILMN_2218856	0.3999	<0.0001	0.6257	<0.0001	1.0256	<0.0001	*CCL3L1*	Chemotaxis, immune response, inflammatory response, negative regulation of cell proliferation	Chemokine activity
ILMN_1682636	0.2815	0.0058	0.718	<0.0001	0.9995	<0.0001	*CXCL2*	Chemotaxis, immune response, inflammatory response	Chemokine activity
ILMN_1747355	0.3699	0.0002	0.589	<0.0001	0.9588	<0.0001	*CCL3L1*	Chemotaxis, immune response, inflammatory response, negative regulation of cell proliferation	Chemokine activity
ILMN_1783593	0.372	0.0039	0.5464	<0.0001	0.9184	<0.0001	*CCL13*	Cell-cell signaling, cellular calcium ion homeostasis, chemotaxis, immune response, inflammatory response, signal transduction	Chemokine activity, signal transducer activity
ILMN_1733259	0.2254	0.0038	0.667	<0.0001	0.8924	<0.0001	*ADORA3*	G-protein coupled receptor protein signaling pathway, activation of adenylate cyclase activity, inflammatory response, regulation of heart contraction, signal transduction	Adenosine receptor activity; G-protein coupled, receptor activity
ILMN_1718552	0.354	0.0038	0.3604	0.0043	0.7143	<0.0001	*CXCL13*	Cell-cell signaling, chemotaxis, elevation of cytosolic calcium ion concentration, immune response, inflammatory response, lymph node development	Chemokine activity
ILMN_2112256	0.4065	<0.0001	0.2456	0.0046	0.6522	<0.0001	*TNFRSF4*	Cellular defense response, immune response, inflammatory response, negative regulation of cytokine secretion, regulation of apoptosis, regulation of protein kinase activity	Protein binding, receptor activity, tumor necrosis factor receptor activity
ILMN_1773245	0.307	0.0004	0.3064	0.0021	0.6134	<0.0001	*CCL3L1*	Chemotaxis, immune response, inflammatory response, negative regulation of cell proliferation	Chemokine activity
ILMN_1703531	0.3809	0.0003	0.2275	0.0056	0.6084	<0.0001	*S1PR3*	G-protein coupled receptor protein signaling pathway, anatomical structure morphogenesis, elevation of cytosolic calcium ion concentration, inflammatory response, inhibition of adenylate cyclase activity by G-protein signaling, positive regulation of cell proliferation, signal transduction	Hydrogen ion transmembrane transporter activity, lipid binding, lysosphingolipid and lysophosphatidic acid receptor activity, receptor activity
ILMN_2320888	0.2758	0.0044	0.2926	0.0039	0.5684	<0.0001	*CXCR4*	G-protein coupled receptor protein signaling pathway, T cell proliferation, activation of MAPK activity, ameboidal cell migration, apoptosis, brain development, chemotaxis, elevation of cytosolic calcium ion concentration, germ cell development, germ cell migration, immune response, inflammatory response, initiation of viral infection, interspecies interaction between organisms, motor axon guidance, neuron migration, patterning of blood vessels, regulation of cell migration, response to hypoxia, response to virus, signal transduction	C-C chemokine receptor activity, C-X-C chemokine receptor activity, actin binding, coreceptor activity, myosin light chain binding, protein binding
ILMN_1810191	0.2519	0.0016	0.305	0.0001	0.5569	<0.0001	*PLA2G4C*		

(*continued on next page*)

Table 1 (***Continued***)

Probe	Comparing T1−Ta		Comparing T2-4−T1		Comparing T2-4−Ta		Gene symbol	Biological process	Molecular function
	Log ratio	*P* value	Log ratio	*P* value	Log ratio	*P* value			
								Arachidonic acid metabolic process, glycerophospholipid catabolic process, inflammatory response, intracellular signaling cascade, lipid catabolic process, metabolic process, parturition	Calcium-independent phospholipase A2 activity, hydrolase activity, phospholipid binding
ILMN_1693014	0.2606	0.0066	0.291	0.0086	0.5516	<0.0001	*CEBPB*	Acute-phase response, anti-apoptosis, embryonic placenta development, fat cell differentiation, immune response, induction of apoptosis, inflammatory response, neuron differentiation, positive regulation of transcription, regulation of interleukin-6 biosynthetic process, regulation of transcription; DNA-dependent, transcription from RNA polymerase II promoter	Protein heterodimerization activity, protein homodimerization activity, sequence-specific DNA binding, transcription activator activity, transcription factor activity
ILMN_1717207	0.3122	<0.0001	0.2212	0.0048	0.5334	<0.0001	*MMP25*	Inflammatory response, metabolic process, proteolysis	Calcium ion binding, metalloendopeptidase activity, peptidase activity, zinc ion binding
ILMN_1678833	0.1588	0.006	0.3101	<0.0001	0.4689	<0.0001	*CCR1*	G-protein signaling; coupled to cyclic nucleotide second messenger, cell adhesion, cell-cell signaling, chemotaxis, cytokine-mediated signaling pathway, elevation of cytosolic calcium ion concentration, immune response, inflammatory response	C-C chemokine receptor activity, protein binding, receptor activity
ILMN_1721762	0.1838	0.0032	0.2808	<0.0001	0.4646	<0.0001	*IL18RAP*	Cell surface receptor linked signal transduction, inflammatory response, innate immune response	Transmembrane receptor activity
ILMN_1747251	0.2954	<0.0001	0.1634	0.0065	0.4588	<0.0001	*LTB4R*	Activation of phospholipase C activity by G-protein coupled receptor protein signaling pathway coupled to IP3 second messenger, cell motion, immune response, inflammatory response, muscle contraction, signal transduction	Leukotriene B4 receptor activity, nucleotide binding, receptor activity
ILMN_2323992	0.1197	0.0071	0.286	<0.0001	0.4057	<0.0001	*CLEC7A*	T cell activation, carbohydrate mediated signaling, cell surface pattern recognition receptor signaling pathway, cell-cell adhesion, defense response to protozoan, detection of yeast, inflammatory response, innate immune response, phagocytosis; engulfment, phagocytosis; recognition, positive regulation of phagocytosis, positive regulation of tumor necrosis factor production, response to molecule of fungal origin	MHC protein binding, binding, opsonin binding, pattern recognition receptor activity, sugar binding, zymosan receptor activity
ILMN_2413508	0.1404	0.0019	0.1739	0.0051	0.3143	<0.0001	*CD97*	Cell adhesion, cell motion, cell-cell signaling, immune response, inflammatory response, neuropeptide signaling pathway	G-protein coupled receptor activity, calcium ion binding, protein binding

Table 2
Correlations between TNFAIP6 expression and other important clinicopathological parameters in urothelial carcinomas (our cohort).

Parameter	Category	Upper urinary tract urothelial carcinoma				Urinary bladder urothelial carcinoma			
		Case no.	TNFAIP6 expression		*P* value	Case no.	TNFAIP6 expression		*P* value
			Low	High			Low	High	
Gender	Male	158	80	78	0.828	216	101	115	0.081
	Female	182	90	92		79	46	33	
Age (y)	<65	138	66	72	0.508	121	59	62	0.759
	≥65	202	104	98		174	88	86	
Tumor location	Renal pelvis	141	69	72	0.606	–	–	–	–
	Ureter	150	79	71		–	–	–	–
	Renal pelvis & ureter	49	22	27		–	–	–	–
Multifocality	Single	278	143	135	0.261	–	–	–	–
	Multifocal	62	27	35		–	–	–	–
Primary tumor (T)	Ta	89	59	30	**<0.001***	84	57	27	**<0.001***
	T1	92	60	32		88	41	47	
	T2−T4	159	51	108		123	49	74	
Nodal metastasis	Negative (N0)	312	161	151	**0.049***	266	136	130	0.177
	Positive (N1−N2)	28	9	19		29	11	18	
Histological grade	Low-grade	56	34	22	0.079	56	32	24	0.224
	High-grade	284	136	148		239	115	124	
Perineural invasion	Absent	321	168	153	**<0.001***	275	139	136	0.362
	Present	19	2	17		20	8	12	
Vascular invasion	Absent	234	130	104	**0.002***	246	129	117	**0.045***
	Present	106	40	66		49	18	31	
Mitotic rate(per 10 high power fields)	<10	173	91	82	0.329	139	78	61	**0.042***
	≥ 10	167	79	88		156	69	87	

* Statistically significant.

3.4. *Prognostic significances of* TNFAIP6 *expression*

The median follow-up period was 31.7 months (range: 1−175.8). There were 52 UBUC (17.6%), and 60 UTUC patients (17.6%) died of the disease; 76 UBUC (25.8%) and70 UTUC patients (20.6%) developed metastatic tumor. We performed univariate and multivariate analyses to assess whether TNFAIP6 staining was significantly associated with cancer metastasis and patients death.

Regarding UTUC (Table 3), 30.0% patients with high TNFAIP6-expressing tumors died of the disease, whereas the mortality was 5.9% for patients with low TNFAIP6-expressing tumors; 34.1% high TNFAIP6-expressing tumors metastasized, whereas it was only 7.1% for low TNFAIP6-expressing tumors. High TNFAIP6 expression was significantly associated with worse DSS (Fig. 3A, $P < 0.0001$) and MFS (Fig. 3B, $P < 0.0001$) in Kaplan −Meier analysis. After demonstrating the predictive value of TNFAIP6 regarding DSS and MFS in univariate analysis, we evaluated its prognostic value for other clinicopathological features (Table 3). This analysis indicated that in addition to TNFAIP6 staining, pathological stage, histological grade, lymph node metastasis, tumor location, multifocality, PNI, and VI were significant predictors of DSS and MFS. After adjusting all significant predictors in univariate analysis, TNFAIP6 expression remained an independent predictor of DSS (hazard ratio [HR]: 2.891, 95% confidence interval [CI]:1.418−5.895; $P = 0.003$) and MFS (HR: 3.803, 95% CI: 1.991−7.264; $P < 0.001$) in multivariate Cox regression analysis.

Regarding UBUC, 26.4% patients with high TNFAIP6-expressing tumors died of the disease and 42.6% showed tumor metastasis, whereas only 8.8% cancer death and tumor metastasis was observed in patients with low TNFAIP6-expressing tumors. Univariate log-rank analysis showed that pathological stage, nodal metastasis, histological grade, mitotic rate, VI, PNI, and TNFAIP6 expression correlated significantly with DSS and MFS (Table 4). Kaplan−Meier curves revealed that high TNFAIP6 expression predicted poor DSS (Fig. 3C, $P < 0.0001$) and MFS (Fig. 3D, $P < 0.0001$). Therefore, to determine whether TNFAIP6 was an independent predictor of DSS and MFS, we performed multivariate analysis including all the significant parameters tested in univariate analysis. Multivariate analysis additionally demonstrated that pathological stage, PNI, mitotic rate, and TNFAIP6 expression status (HR: 2.175, 95% CI: 1.149−4.117; $P = 0.017$) significantly correlated with DSS; pathological stage, mitotic rate, and TNFAIP6 expression status (HR: 3.845, 95% CI: 2.105 −7.025; $P < 0.001$) were considerably associated with MFS (Table 4).

4. Discussion

UC represents a clinically heterogeneous cancer. Patients with the same tumor stage and grade may have

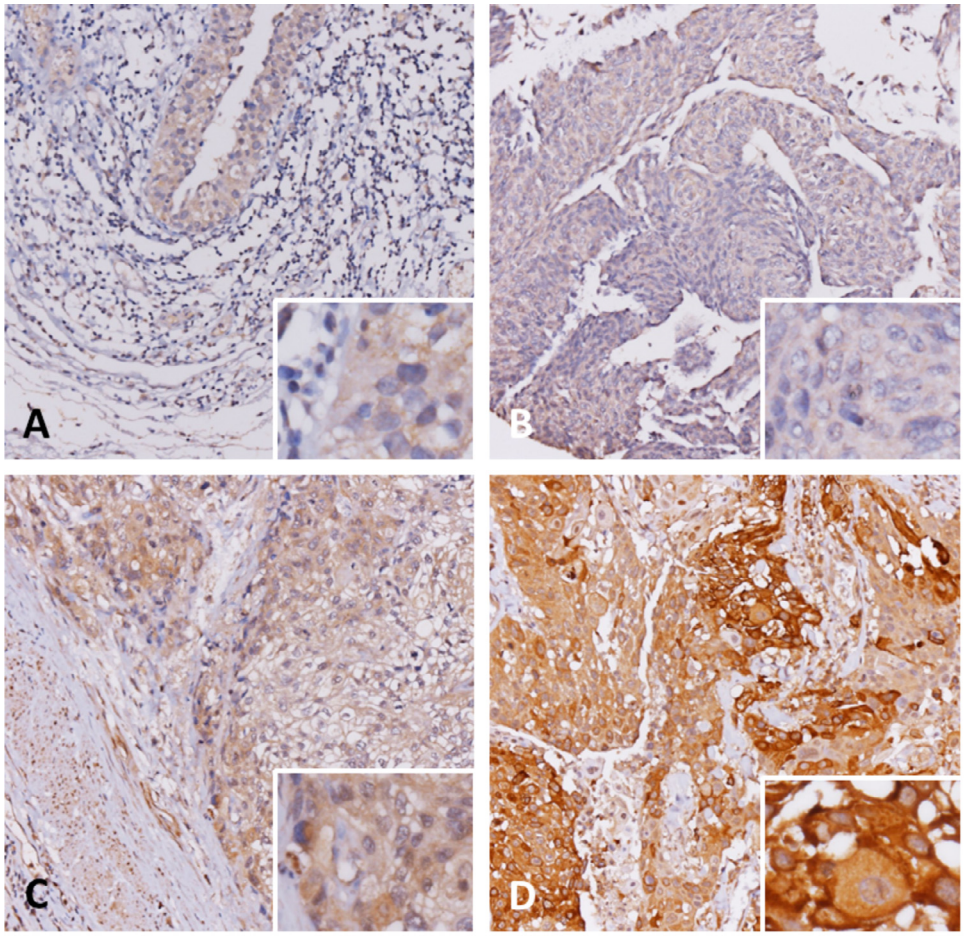

Fig. 2. TNFAIP6 immunohistochemistry. The representative sections of (A) normal urothelium (B) noninvasive urothelial carcinoma, (C) superficially invasive urothelial carcinoma, and (D) high-stage urothelial cracinoma, respectively, demonstrating a stepwise increment in TNFAIP6 expression. (original magnification × 400).

diverse outcomes [2–6]. Therefore, identification of prognostic biomarkers may improve the accuracy of risk stratification. In this study, we confirmed that tissue immunoexpression of TNFAIP6 was an independent predictor of DSS and MFS in UCs. High TNFAIP6 expression was significantly related to aggressive pathological characteristics, suggesting its roles in tumor development and progression. In addition, high TNFAIP6 immunoreactivity indicated 2.891 times and 2.175 times increase in the risk of cancer specific deaths in UTUC and UBUC, respectively; it also showed 3.803 times and 3.845 times increase in the risk of metastatic tumor dissemination in UTUC and UBUC, respectively. These observations suggested that TNFAIP6 immunoexpression may be used with standard clinical and pathological prognostic factors for better risk stratification for UC.

TNFAIP6, located on human chromosome 2q23.3, was initially recognized as the sixth gene product induced by TNF in human fibroblasts [21]. *TNFAIP6* contains a hyaluronan-binding LINK domain and a CUB domain that interacts with other proteins or carbohydrates [15,22]. It is an inflammation-associated protein that has been shown to be up-regulated by proinflammatory cytokines such as TNFα, interferon-γ, prostaglandin E2, interleukin-1, and lipopolysaccharide [15,23]. In contrast, TNFAIP6 expression is suppressed by anti-inflammatory cytokines, for instance interleukin-4 or interleukin-10. Not unexpectedly, TNFAIP6 is detected in many inflammatory diseases, including osteoarthritis, systemic lupus erythematosus, rheumatoid arthritis, inflammatory bowel disease, and coronary artery disease [15,16]. It is also produced in inflammation-like processes, such as ovulation and cervical ripening [15,23].

Chronic inflammation is an important risk factor for the development of UB cancer. Other factors involved in inflammation-associated bladder cancer, include infections, chronic mechanical and chemical irritation, and germline inflammatory variants [8,9,13]. Through the recruitment of inflammatory cells, including macrophages, regulatory T cells, neutrophils, and signaling molecules such as TNF and cytokines, chemokine/chemokine receptors, and transcription factors in tumor inflammatory microenvironment

Table 3
Univariate log-rank and multivariate analyses for disease-specific and metastasis-free Survivals in upper urinary tract urothelial carcinoma (our cohort).

Parameter	Category	Case no.	Disease-specific survival					Metastasis-free survival				
			Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis		
			No. of event	*P* value	R.R.	95% C.I.	*P* value	No. of event	*P* value	R.R.	95% C.I.	*P* value
Gender	Male	158	28	0.8286	–	–	–	32	0.7904	–	–	–
	Female	182	33		–	–	–	38		–	–	–
Age (years)	<65	138	26	0.9943	–	–	–	30	0.8470	–	–	–
	≥65	202	35		–	–	–	40		–	–	–
Tumor side	Right	177	34	0.7366	–	–	–	38	0.3074	–	–	–
	Left	154	26		–	–	–	32		–	–	–
	Bilateral	9	1		–	–	–	0		–	–	–
Tumor location	Renal pelvis	141	24	**0.0079***	1	–	0.536	31	0.0659	–	–	–
	Ureter	150	22		0.761	0.405–1.429		25		–	–	–
	Renal pelvis and ureter	49	15		1.309	0.363–4.721		14		–	–	–
Multifocality	Single	273	48	**0.0026***	1	–	0.268	52	**0.0127***	1	–	**<0.001***
	Multifocal	62	18		1.991	0.589–6.723		18		2.315	1.331–4.028	
Primary tumor (T)	Ta	89	2	**<0.0001***	1	–	0.147	4	**<0.0001***	1	–	0.122
	T1	92	9		3.191	0.677–15.045		15		1.893	0.584–6.137	
	T2–T4	159	50		3.484	0.753–16.115		51		2.969	0.958–9.208	
Nodal metastasis	Negative (N0)	312	42	**<0.0001***	1	–	**<0.001***	**55**	**<0.0001***	1	–	**<0.001***
	Positive (N1–N2)	28	19		5.667	3.066–10.475		**15**		3.169	1.727–5.813	
Histological grade	Low grade	56	4	**0.0215***	1	–	**0.011***	3	**0.0027***	1	–	0.051
	High grade	284	57		3.586	1.341–9.590		67		2.220	0.998–4.938	
Perineural invasion	Absent	321	50	**<0.0001***	1	–	**0.002***	61	**<0.0001***	1	–	0.126
	Present	19	11		3.154	1.503–6.617		9		1.785	0.850–3.750	
Vascular invasion	Absent	234	24	**<0.0001***	1	–	0.083	26	**<0.0001***	1	–	**0.001***
	Present	106	37		1.703	0.932–3.110		44		2.799	1.541–5.083	
Mitotic rate (per 10 high power fields)	<10	173	27	0.167	–	–	–	30	**0.0823**	–	–	–
	≥ 10	167	34		–	–	–	40		–	–	–
TNFAIP6 expression	Low	170	10	**<0.0001***	1	–	**0.003***	12	**<0.0001***	1	–	**<0.001***
	High	170	51		2.891	1.418–5.895		58		3.803	1.991–7.264	

* Statistically significant**.**

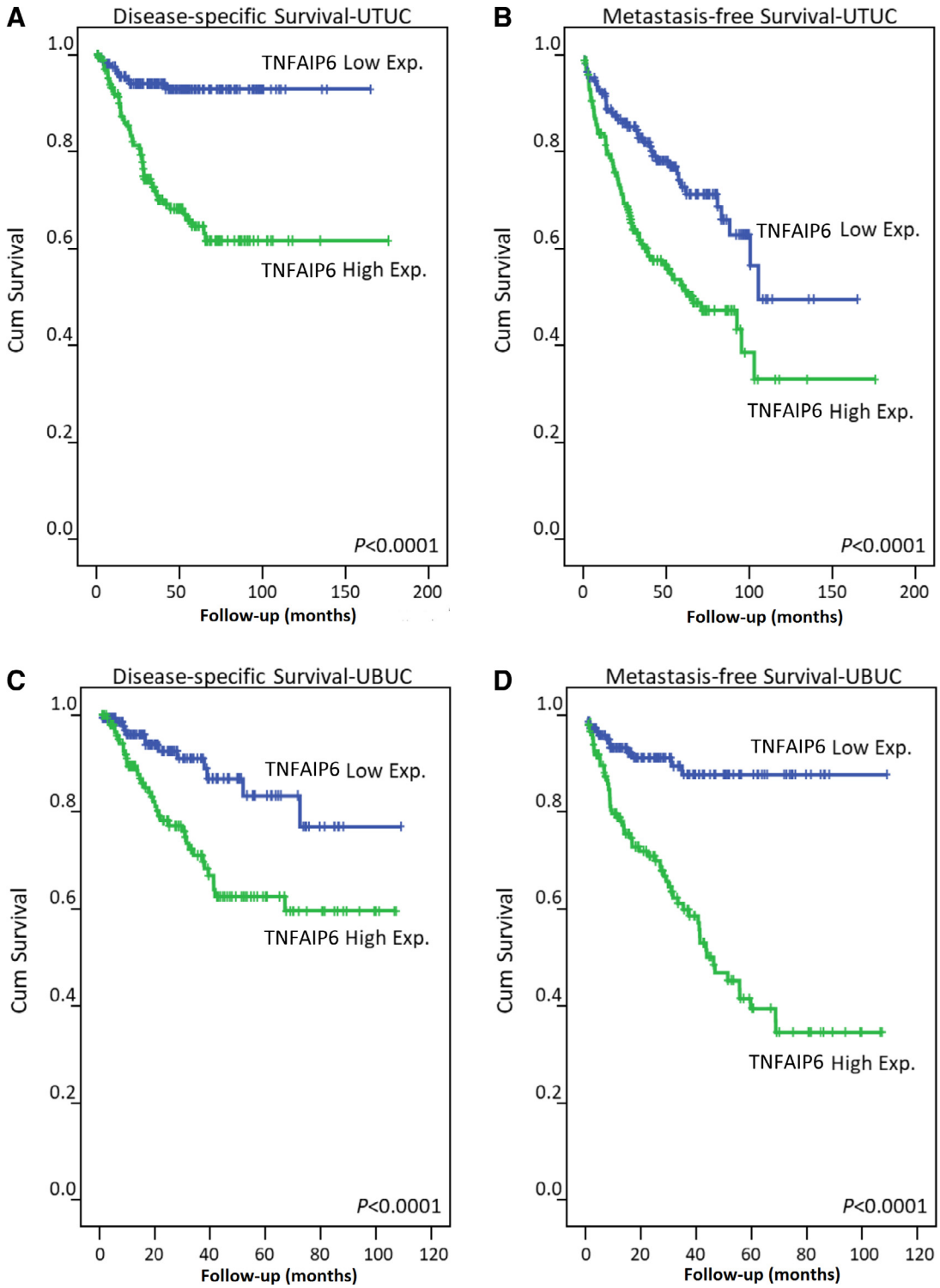

Fig. 3. Kaplan–Meier plots show that TNFAIP6 overexpression is associated with significant prognostic impact on disease-specific survival and metastasis-free survival of patients with UTUC (A and B, respectively) and UBUC (C and D, respectively). Abbreviations: UBUC = urinary bladder urothelial carcinoma; UTUC = upper urinary tract urothelial carcinoma.

may contribute to bladder cancer formation and progression [8,9,13]. Furthermore, increased ECM protein's deposition and crosslinking in the tumor microenvironment can promote tumor-associated inflammation, proliferation, angiogenesis, migration, and invasion [24–26]. TNFAIP6 can bind to several ECM components, such as glycosaminoglycan, hyaluronan, and fibronectin [15,27]. Owing to these interactions, TNFAIP6 is associated with ECM remodeling and stabilization [27]. Therefore we selected TNFAIP6 for further study.

Table 4
Univariate log-rank and multivariate analyses for disease-specific and metastasis-free survivals in urinary bladder urothelial carcinoma (our cohort).

Parameter	Category	Case no.	Disease-specific survival					Metastasis-free survival				
			Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis		
			No. of event	*P* value	R.R.	95% C.I.	*P* value	No. of event	*P* value	R.R.	95% C.I.	value
Gender	Male	216	41	0.4446	–	–	–	60	0.2720	–	–	–
	Female	79	11		–	–	–	16		–	–	–
Age (y)	<65	121	17	0.1136	–	–	–	31	0.6875	–	–	–
	≥65	174	35		–	–	–	45		–	–	–
Primary tumor (T)	Ta	84	1	**<0.0001***	1	–	**<0.001***	4	**<0.0001***	1	–	**0.002***
	T1	88	9		4.456	0.488–40.682		23		3.016	0.896–10.153	
	T2–T4	123	42		20.269	2.373–173.159		49		5.274	1.594–17.450	
Nodal metastasis	Negative (N0)	266	41	**0.0002***	1	–	0.535	61	**<0.0001***	1	–	0.084
	Positive (N1–N2)	29	11		1.253	0.614–2.556		15		1.730	0.929–3.224	
Histological grade	Low grade	56	2	**0.0013***	1	–	0.767	5	**0.0007***	1	–	0.737
	High grade	239	50		1.257	0.277–5.708		71		1.369	0.497–3.769	
Perineural invasion	Absent	275	44	**0.0001***	1	–	**0.029***	66	**0.0007***	1	–	0.063
	Present	20	8		2.534	1.100–5.839		10		2.036	0.963–4.306	
Vascular invasion	Absent	246	37	**0.0024***	1	–	0.124	54	**0.0001***	1	–	0.657
	Present	49	15		1.711	0.864–3.387		22		1.143	0.633–2.065	
Mitotic rate (per 10 high power fields)	<10	139	12	**<0.0001***	1	–	**0.018***	23	**<0.0001***	1	–	**0.039***
	≥10	156	40		2.276	1.153–4.493		53		1.736	1.029–2.930	
TNFAIP6 expression	Low	147	13	**<0.0001***	1	–	**0.017***	13	**<0.0001***	1	–	**<0.001***
	High	148	39		2.175	1.149–4.117		63		3.845	2.105–7.025	

* Statistically significant.

Although TNFAIP6 has been extensively studied with respect to inflammatory disease, its role in cancer remains largely unexplored [15,16]. RNA expression profiling analysis showed that*TNFAIP6* mRNA level was significantly higher in the peripheral blood of patients with colon cancer [28]. Immunohistochemical staining showed higher TNFAIP6 expression in colon cancer specimens than in adjacent normal tissues [17]. High TNFAIP6 expression predicted poor overall and recurrence-free survival rates in patients with colon cancer [17,18]. In ovarian cancer, TNFAIP6 was up-regulated in higher T stage and metastatic tumor. High TNFAIP6 expression correlated with poor prognosis [17,29]. Genome-wide cDNA expression data analysis showed that TNFAIP6 was also up-regulated in breast, lung, gastric, oral, and pancreatic cancer [17]. However the clinical significance is still being investigated.

Our results showed that high TNFAIP6 expression correlated significantly with aggressive pathological features, including advanced stage, VI, PNI, lymph node metastasis, and high mitotic activity, indicating its significance in UC progression. For effectively treating nonmuscle invasive bladder cancer, identification of high-risk patients who may progress to muscle invasive bladder cancer is critical [2,3]. We observed that TNFAIP6 immunoexpression may be a biomarker for identifying these patients, as significantly high TNFAIP6 expression was observed in muscle invasive bladder cancer and high-grade UBUC. Tumor metastasis is the major reason of UC associated death [1−5]. There is a marked survival benefit of cisplatin-based chemotherapy or immunotherapy for patients with locally advanced or metastatic UC [2,4], highlighting the need of early recognition of the metastatic potential. Our results demonstrated that UC patients with aggressive pathological characteristics and high TNFAIP6 expression were more likely to metastasize distantly. These observations will expedite early treatment and detection of UC. Patients with TNFAIP6-overexpressing UCs may receive more aggressive management, such as adjuvant/neoadjuvant systemic chemotherapy or immunotherapy, and radical operation with regional/extended lymph node dissection.

However, our study has certain limitations. First, this is a retrospective single center study. Second, we could not elucidate the precise signaling pathways and molecular mechanism underlying tumor progression, which resulted in poor outcomes in patients with TNFAIP6-overexpressing UC. Third, the immunohistochemical techniques used had inherent variability. In this study we used the H-score because of its excellent correlation with results of western blotting [30]. Finally, prospective studies are warranted to confirm our results.

5. Conclusions

We demonstrated the independent predictive value of TNFAIP6 on patient survival and cancer metastasis in UTUC and UBUC. Incorporating TNFAIP6 immunostaining in current pathological examinations can assist clinicians in identifying high-risk patients to facilitate individualized medical decision-making and therapy.

Conflict of interest

The authors have declared that no conflict of interest exists.

Acknowledgment

The authors are grateful to the BioBank at Chi Mei Medical Center for providing tumor samples.

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