Indoleamine-2,3-dioxygenase-1 expression predicts poorer survival and up-regulates ZEB2 expression in human early stage bladder cancer

Yuh-Shyan Tsai, M.D., Ph.D.[a],[*],[1], Yeong-Chin Jou, M.D.[b],[1], Hsin-Tzu Tsai, M.S.[a],
Ian-Seng Cheong, M.D.[b], Tzong-Shin Tzai, M.D.[c]

[a] Department of Urology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan
[b] Department of Urology, Chia-Yi Christian Hospital, Chia-Yi, Taiwan
[c] An-Nan Hospital, China Medical University, Tainan, Taiwan

Abstract

Purposes: Indoleamine-2,3-dioxygenase-1 (IDO1) is a key enzyme of tryptophan metabolism which regulates T cell function in immune cells and little is known about the role of IDO1 expression in bladder cancer cells. The study is aimed to evaluate the clinical relevance of IDO1 expression in human bladder urothelial carcinoma (UC).

Materials and Methods: One hundred and sixty paraffin-embedded UC tissues (130 bladder, 30 upper urinary tract) and 47 adjacent normal tissues were retrieved for IDO1 immunostaining. Urine samples from UC and non-UC patients were collected before surgery for measuring the concentration of tryptophan and its metabolites. Clinicopathological correlates of IDO1 expression and the prognostic values in human bladder cancer were explored. External validation was performed with 4 published bladder cancer datasets, as well as in vitro studies.

Results: As compared with normal adjacent tissues, UC exhibited a higher frequency of IDO1 expression (chi-square, $P = 0.0005$). IDO1 expression is an independent poor prognostic factor for disease progression [hazard ratio and 95% confidence interval, 3.80 (1.46−9.86), $P = 0.006$], which is associated with decreased number of intratumoral infiltrating CD8+ lymphocyte (unpaired t test, $P = 0.026$). External validation showed that patients with higher IDO1 expression exhibit decreased disease-specific survival than those with lower IDO1 expression. Furthermore, IDO1 expression correlated positively with the expression of several EMT markers, including ZEB2, fibronectin and vimentin. The in vitro T24 cell subline demonstrated that IDO1 expression can up-regulate ZEB2 expression probably through miR-200c signaling.

Conclusion: IDO1 expression predicts poorer survival and up-regulates ZEB2 expression in human bladder cancer. © 2019 Elsevier Inc. All rights reserved.

Keywords: Bladder neoplasm; Indoleamine-2,3-dioxygenase-1; Epithelial-mesenchymal transition; Prognosis

1. Introduction

Bladder cancer is the fourth most common cancer in men and the ninth in women in western countries, and the highest incidence rates in men are seen in countries from Southern Europe, North America, Northern Africa and Western Asia [1]. At initial diagnosis, approximately 70% to 80% of bladder cancer cases are nonmuscle invasive. Most of these (about 90%) easily recur, and about 15% may progress to muscle-invasive diseases despite endoscopic resection and adjuvant intravesical therapy [2]. In contrast, 20% to 30% of bladder cancer cases are muscle-invasive, advanced, or metastatic tumors upon diagnosis. Radical cystectomy or bladder-sparing tri-modality therapy remains the main choices for patients with muscle-invasive disease [3]. Despite of systemic platinum-based chemotherapy, half of these die of metastatic disease within 5 years. Until 2016, there was limited therapeutic progress in

*Corresponding author. Tel.: +886-6-235-3535, ext: 5251; fax: +886-6-238-3678.
E-mail addresses: youh@mail.ncku.edu.tw, s9889104330@gmail.com (Y.-S. Tsai).
[1]Both the authors contributed equally.

advanced or metastatic diseases. During the recent 2 years, 5 immune checkpoint inhibitors have gained the Food and Drug Administration's approval for treating advanced or metastatic urothelial carcinoma [4], there is still 70% to 80% patients presenting with primary, adaptive, and acquired resistance to cancer immunotherapy [5]. Actually, the interactions between the cancer cells and immune system are dynamic and continuously evolving from the beginning of a cancer cell to the metastasis, from initial diagnosis to after treatment. Therefore, it is still necessary to explore the significance of some promising molecules in urothelial carcinoma, which might interact with those factors involving the immune system.

Indoleamine-2,3-dioxygenase (IDO) is a heme-containing intracellular enzyme that initiates the first and rate-limiting step of tryptophan metabolism [6]. This family includes IDO1, IDO2, and tryptophan dioxygenase 2 and can catalyze the oxidative degradation of L-tryptophan to N-formyl kynurenine, then 3-hydroxyanthranilic acid (3-HAA) and kynurenine. In human malignancies, IDO was expressed not only by dendritic cells in tumor draining lymph nodes, but also by tumor cells themselves, which was associated with a worse prognosis in melanoma patients [7]. Not only enzymatic activity involving in the immune responses (i.e., recruitment of a Treg phenotype, inactivation of cytotoxic T cell, and NK cell activity) [8], but also IDO protein exhibits its nonenzymatic regulation involved in the noncanonical NF-κB pathway [9]. Also, Tumor-expressed IDO can recruit myeloid-derived suppressor cells in Treg-dependent manner [10]. In human bladder cancer, IDO1 expression is one of immune inhibitory molecules associated with T-cell inflamed subtypes according to data mining from TCGA database [11]. Despite this, little is known regarding the significance of IDO1 expression in human bladder cancer.

The process of epithelial-mesenchymal-transition (EMT) plays a pivotal role during metastasis [12]. ZEB1/2 is one of master EMT-inducing transcription factors that orchestrate or coordinate the complex signal involved the EMT program. ZEB proteins can selectively bind to an E-box element of promoter region of target genes (e.g., E-cadherin) though zinc-finger clusters, contributing to EMT process [13]. Also, knocking down ZEB2 expression can suppress cell proliferation, invasion, and migration in glioma cells [14]. Several microRNAs were demonstrated to be able to regulate the ZEB protein expression, including miR-200 [13], and miR-29a [15]. In the current study, we demonstrated that tumor-expressed IDO1 protein is an independent prognostic factor for bladder cancer progression and up-regulate the ZEB2 expression though miR-200c signaling.

2. Materials and methods

2.1. Patient population and study samples

The approval and institutional oversight of the Institutional Review Board for the Protection of Human Subjects at both of Chia-Yi Christian Hospital (IRB-101014) and National Cheng Kung University Hospital (ER-95-49 and A-ER-102-381) were obtained in the current study. Formalin-fixed paraffin-embedded tumor specimens from urothelial cancer patients were retrospectively retrieved from the archives of both hospitals. All patients that received endoscopic resection of bladder tumor, cystectomy, or nephroureterectomy were included in the study. The tumor was categorized according to the 2007 TNM staging and the 2004 WHO grade system. All the patients were treated followed according to the bladder cancer or upper urinary tract treatment consensus, modified from NCCN Clinical Practice Guidelines in Oncology.

2.2. Immunohistochemistry (IHC)

Serial 5-μm sections were cut for immunohistochemistry (IHC) as per our previous study [16]. Briefly, tissue sections were deparaffinized, rehydrated, autoclaved, and sequentially treated with 3% H_2O_2 in methanol, and skimmed milk in phosphate buffer saline. Nonspecific background staining was reduced by preincubating with 0.3% bovine serum albumin. The primary antibodies were mouse antihuman IDO (MAB5412, Merck Millipore, Billerica, MA) and anti-CD8 (DAKO, Glostrup, Denmark; dilution, 1:200). All stains included a negative control using mouse isotype IgG1 (DAKO, Cytomation), and a positive control was obtained from prior samples with known positive staining (placenta for IDO). After incubation with secondary antibodies for 1 hour, the immunostaining was developed with a BioGenex Super Sensitive Polymer HRP IHC System kit and then counterstained with hematoxylin. Samples were analyzed blindly by 1 pathologist.

After immunostaining, papillary lesions, or mucosal portions for superficial tumors and infiltrating portions or tumor nesting for solid, muscle-invasive tumors were mainly evaluated for IDO1 expression status. As for IDO1 immunostaining, any tumor specimens exhibiting more than 25% immunoreactive tumor cells within 1 high power field were thought as strongly positive (++), those 5% to 25% or weak intensity as weak positive (+), and less than 5% as negative tumors. As for the significance of CD8-positive tumor-infiltrating lymphocytes (CD8^{+}TILs), the average number was manually determined from 10 random 0.0328-mm^2 digital images captured under high power field (×320). All counts were repeated 3 times by the same pathologist, and the average of the repeat counts was used for statistical analyses.

2.3. Cells, plasmids, and reagents

Several human urothelial cancer cell lines were used in the current study. Three human bladder cancer cell T24 sublines were kindly provided by professor Hsieh, including T24-P (primarily subcultured from orthotropic bladder site), T24-L (primarily subcultured from lung metastatic

site), and T24-B (primarily subcultured from bony metastatic site). All of these cells were maintained in DME medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 μg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO_2.

Three IDO1 shRNAs were obtained from The RNAi Consortium (TRC) shRNA Library, including TRCN0000056744, TRCN0000056745, and TRCN0000056746 (abbreviated as sh-IDO1 #44, #45, and #46). For plasmid transfection, the protocol was same with the previous study [17]. While cells were seeded in plates with 70% to 80% confluence, the transfection was carried out using polyethylenimine (Polysciences Inc., Warrington, PA) according to the manufacturer's instructions. The microRNA inhibitor for miR-200c (hsa-miR-200c-5p; ID, MH12741) was purchased from Ambion (Austin, TX).

2.4. Western blotting

A Western blot was performed as per our previous study [17]. Whole cell lysate protein was obtained from cells at 80% to 90% confluence. Thirty micrograms of protein from each sample was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto nitrocellulose filter, and subsequently immunoblotted with anti-IDO antibody (GTX113753, Genetex), or anti-ZEB2 antibody (SAB2108744, Sigma). The β-actin was used as a control.

2.5. Quantitative RT-PCR

Total ribonucleic acid (RNA) was harvested from T24 sublines at 80% to 90% confluence for further assays. Total RNA was extracted using the TRIzol (Invitrogen, CA) method according to the manufacturer's protocol and then reverse transcribed with High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, CA). The resulting cDNA was used for PCR in triplicate and data collections were performed in a Step-One Plus Real Time PCR system (Applied Biosystems, CA). All samples were amplified simultaneously in duplicates in a one-assay run. The primers for human IDO1, IDO2, tryptophan dioxygenase 2, ZEB2, and microRNAs were used (Supplementary Table 1). The $-\Delta\Delta C_t$ method was utilized to measure relative changes in mRNA levels examined by the quantitative reverse transcriptase PCR (qRT-PCR) experiments, after normalizing the transcript levels of each gene by the levels of β-actin and RUN6B as an internal control.

2.6. High-performance liquid chromatographic (HPLC) measurement for 3-HAA and tryptophan

For measuring both tryptophan and 3-HAA levels in urine, the urine samples were collected before surgery via catheterization. These urine samples were obtained from different patients whose tissue samples were used for immunostaining described as above. The concentration of

tryptophan and 3-HAA in urine was determined with high-performance liquid chromatographic (HPLC) method according to the manufacture's protocol (Mission Biotech, Taipei, Taiwan). The collected urine samples were centrifuged at 3,000 g and the supernatant were stored at −80°C for further HPLC measurement. The 20 μl urine supernatant was diluted with 180 μl of methanol at −20°C for 1 hour and then centrifuged at 3,000 g for 15 minutes. All chromatographic procedures were performed in 37°C, with column (Phenomenex Synergi 4 μm Fusion, RP-80A), AB Sciex Instruments QTRAP 5500, and injection volume of 5 μl. Two standards stock solutions were prepared in ethanol and stored at −20°C. The standard agents were 3-HAA (M.W. 153.1) and tryptophan (M.W. 204.2) (Sigma). A fresh solution of all standards was used to prepare mixed calibrators and to serial dilutions to prepare a standard curve range. The quantitative method was Multiple Point External Standard.

2.7. Cell proliferation assay

An MTT assay was used for evaluation of cell proliferation assay. In brief, cells were seeded in a 96-well plate at 5,000 cells per well and cultivated overnight in 2% fetal bovine serum-containing medium. When control wells were near 80% to 90% confluence, cell viability was determined by adding 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma, St. Louis, MO) into each well (a final concentration of 0.05%) and incubated 4 hours at 37°C. One hundred and fifty microliter of dimethyl sulfoxide was added and the plates were incubated for 3 minutes. The optical density (O.D.) of each well was determined using a microplate reader at a wavelength of 570 nm. The percentage of cell viability is calculated by the O.D. value of treated cells normalized with the O.D. value of control cells. The growth index was calculated by the formula: (%48 h or %72 h/%24 h).

2.8. Kynurenine enzyme-linked immunosorbent assay

Before the addition of MTT agent, the media were collected for kynurenine measurement. In brief, the supernatant of the collected media after acylation for 90 minutes at 37°C was determined by antibody-capture bioassay with a kynurenine ELISA kit (BAE-2200, LDN, Nordhorn, Germany) according to the manufacturer's protocols.

2.9. Data mining

For analyzing the correlation of IDO1 expression with EMT markers, 4 human urothelial carcinoma dataset GSE13507 [18], GSE31684 [19], GSE32548 [20], and GSE48075 [21] were used. Processed data were downloaded from NCBI GEO, and log2 data for individual probes were utilized for calculating the correlation

coefficient using Pearson's method (Graphpad Software 6th version, San Diego, CA).
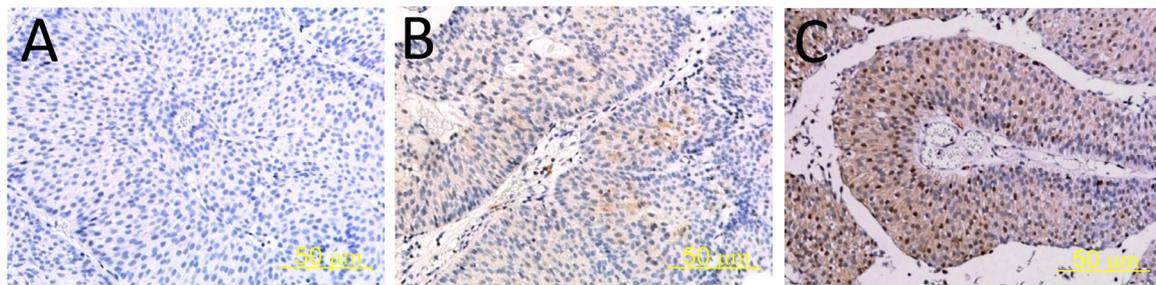
2.10. Statistical analysis

The clinicopathological correlation with the IDO family expression was analyzed from all the studied subjects. The clinical outcomes of patients were analyzed. Recurrence-free survival, progression-free survival, and disease-specific survival were calculated from surgery to the date of the first documented tumor recurrence in the urinary bladder, or tumor progression into muscle-invasive diseases, metastases, or cancer death, respectively. Statistical analysis was performed using Statistical Package for Social Sciences, version 12.0, software (SPSS). The relationships between IDO1 expression, and clinicopathological factors were analyzed with chi-square

test or unpaired t test. The prognostic significance of IDO1 expression pattern and other clinicopathological variables on disease recurrence or progression were analyzed with Kaplan-Meier plots, the log-rank test, and the multivariate Cox regression model. All P values reported are 2-sided and considered significant if $P < 0.05$.

3. Results

3.1. IDO1 expression is higher in the urothelial tumor than normal adjacent tissue

A total of 130 human bladder cancer, 30 upper tract urothelial carcinoma, 26 normal adjacent urothelial tissue, and 21 renal cortex were immunostained for IDO expression (Fig. 1A−C). Tumor specimens of bladder cancer and

Specimen	IDO		
	-	+	++
Bladder UC	55	25	50
UTUC	6	4	20
Nr. Uro Epi	15	4	7
Renal cortex	16	2	3

* Bladder UC and UTUC *versus* adjacent normal tissue , $p = 0.005$ (negative and + *vs.* ++)
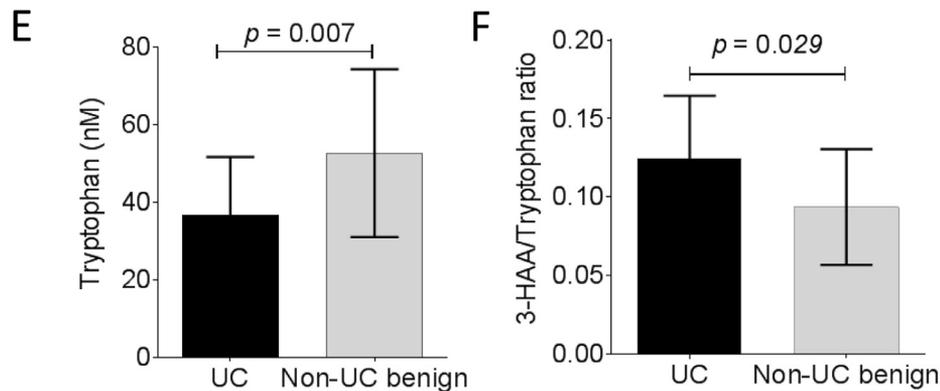
Fig. 1. IDO1 expression in human urothelial tumors and adjacent normal tissues. (A−C) Immunohistochemical staining for IDO1 protein. A, negative expression; B, weak positive; C, strong positive. (Scale bar = 50 μm, ×320). (D) IDI1 expression in urothelial carcinoma of upper tract and bladder, as well normal adjacent renal cortex and uroepithelia. (E, F) HPLC study for urine tryptophan and 3-HAA. IDO1 = indoleamine-2,3-dioxygenase-1; Nr Uro Epi = normal urothelial epithelia; UC = urothelial carcinoma; 3-HAA, 3-hydroxyanthranilic acid.

upper tract UC exhibit more strong staining than normal adjacent tissues (chi-square, $P = 0.0005$; Fig. 1D). The urine tryptophan and 3-HAA concentration were measured using HPLC assays from a total of 41 patients (25 UC and 16 non-UC benign disease). The result showed the urine tryptophan concentration is lower in the UC patients than those in the non-UC benign patients (unpaired t-test, $P = 0.007$) and the urine 3-HAA/tryptophan ratio is higher in the UC patients than those in the non-UC benign patients (unpaired t-test, $P = 0.029$; Fig. 1E and F).

3.2. Association of IDO1 expression with clinicopathological parameters

One hundred and eight of 130 (83%) human bladder tumors can be analyzed for association between IDO1 expression and clinicopathological parameters (Table 1). Overall, there were 73(67.6%) male and 35 (32.4%) female patients in this cohort. There were 45 (41.7%) null, 22 (20.3%) weak, and 41 (38.0%) strong IDO1 expression. Patients with previous history of UC in any site exhibit lower strong IDO1 expression than those without UC

history ($P = 0.024$, chi-square test). Except for the UC history, there was lack of any association between IDO1 expression and other clinicopathological factors, including age, the presence of hemodialysis due to end stage renal disease, tumor number, morphology, grade, and pathological stage (all P values > 0.05; Table 1).

3.3. Prognostic values of IDO1 expression in bladder tumors

During follow-up (median, 45 months), there were 64 (59.3%) recurrences, 24 (22.2%) progressions, and 28 (25.9%) death. Patients with strong IDO1-expressing tumors had higher frequency of disease progression than did those with null or weak IDO-expressing tumors ($P = 0.03$, chi-square test). There was no correlation between IDO1 expression and disease recurrence ($P = 0.57$, chi-square test; $P = 0.450$, log-rank test; Fig. 2A).

In terms of tumor progression, univariate analyses demonstrated that tumor morphology [hazard ratio (HR) and 95% confidence interval (CI), 2.94 (1.21−7.18), $P = 0.018$], tumor stage [HR and 95% CI, 3.11 (1.76−5.49), $P = 0.001$]

Table 1
Clinicopathological correlates of IDO1expression in 108 human bladder urothelial tumors

| Characteristics | Pts. no. (%) | Negative | IDO1 localization | | P* | P** |
			+	++		
Total	108	45	22	41		
Age, mean, SD (y)	68.0 ± 11.6	67.5 ± 11.0	68.9 ± 11.5	66.3 ± 12.9	0.711	0.492
Gender						
Male	73 (67.6)	32	15	26	0.745	0.468
Female	35 (32.4)	13	7	15		
History of UC						
No	60 (55.6)	22	10	28	0.090	0.024
Yes	43 (39.8)	21	11	11		
Unknown	5 (4.6)	2	1	2		
ESRD on dialysis						
No	88 (81.5)	34	22	32	0.041	0.473
Yes	20 (18.5)	11	0	9		
Multiplicity						
Single	31 (28.7)	11	10	10	0.127	0.425
Multiple	74 (68.5)	33	11	30		
Unknown	3 (2.8)	1	1	1		
Morphology						
Papillary	82 (75.9)	35	15	32	0.623	0.535
Non-papillary	22 (20.4)	9	6	7		
Unknown	4 (3.7)	1	1	2		
Tumor grade						
Low	13 (12.0)	6	3	4	0.828	0.540
High	95 (88.0)	39	19	38		
Tumor stage						
Ta	45 (41.7)	20	10	15	0.731	0.527
T1	43 (39.8)	16	10	17		
T2	19 (17.6)	9	2	8		
T3 at least	1 (0.9)	0	0	1		

* One hundred and eight patients with bladder tumors, analyzed with one-way ANOVA test.
** One hundred and eight patients with IDO1 (−) and (+) vs. IDO1 (++) expression, analyzed with chi-square test or Fisher's exact test.CIS = carcinoma in situ; TIL = tumor-infiltrating lymphocytes; UC = urothelial carcinoma.
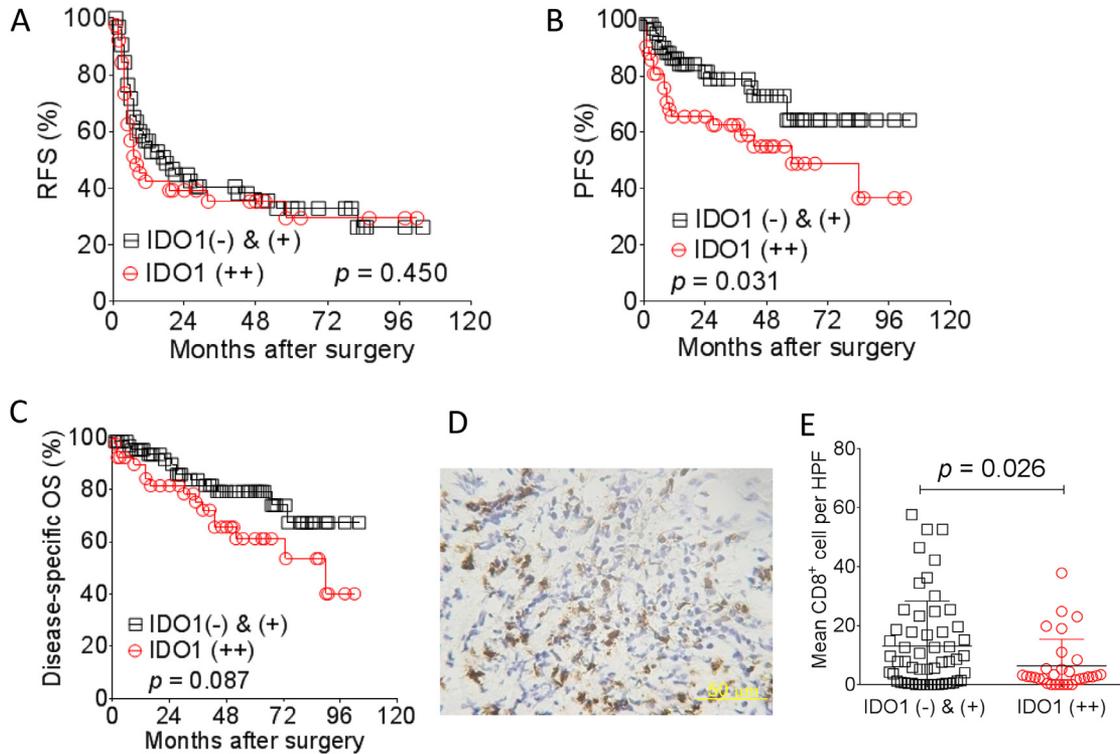
Fig. 2. Patients' outcome according to IDO1 expression. (A) recurrence-free survival according to IDO1 expression, (B) progression-free survival according to IDO1 expression, (C) disease-specific overall survival according to IDO1 expression, (D) immunohistochemical staining for CD8 expression (scale bar = 50 μm, ×320), (E) number of tumor-infiltrating CD8+ lymphocytes according to IDO1 expression.

and IDO1 expression [HR (95% CI), 2.81(1.23−6.44), $P = 0.014$] were significantly associated with progression-free survival, and tumor multiplicity is a borderline significant predictor [HR (95% CI), 3.94 (0.92−16.7), $P = 0.064$]. Multivariate analysis showed IDO1 expression and the other 3 parameters (tumor multiplicity, morphology, and tumor stage) were independent prognostic factors for tumor progression (Table 2 and Fig. 2B).

In term of disease-specific overall survival (DSOS), univariate analyses demonstrated that tumor morphology [HR

Table 2
Univariate and multivariate analyses of variables associated with survival in 108 bladder cancer patients

Variables[a]	PFS		DSOS	
	HR (95% CI)	P	HR (95% CI)	P
Univariate analysis				
Gender	0.81 (0.34−1.97)	0.645	0.96 (0.43−2.13)	0.924
History of UC	0.90 (0.39−2.06)	0.802	1.35 (0.64−2.85)	0.426
ESRD	0.50 (0.07−3.77)	0.504	1.69 (0.58−4.95)	0.338
Multiplicity	3.94 (0.92−16.7)	0.064	1.42 (0.57−3.50)	0.452
Morphology	2.94 (1.21−7.18)	0.018	3.33 (1.48−7.52)	0.004
Grade	29.2 (0.35−2455)	0.135	27.9 (0.43−1813)	0.118
Stage	3.11 (1.76−5.49)	0.001	5.17 (2.78−7.59)	0.001
IDO1 expression	2.81 (1.23−6.44)	0.014	1.92 (0.91−4.03)	0.087
Multivariate analysis[b]				
Multiplicity	9.87 (1.29−75.6)	0.028	1.68 (0.59−4.76)	0.330
Morphology	3.23 (1.03−10.2)	0.045	2.91 (1.09−7.77)	0.033
Stage	2.29 (1.11−4.71)	0.025	3.76 (1.79−7.90)	0.001
IDO1 expression	3.80 (1.46−9.86)	0.006	2.58 (1.12−5.92)	0.025

[a] Variables include gender (male vs. female), previous history of urothelial carcinoma tumor site (no vs. yes), ESRD on dialysis (no vs. yes), multiplicity (single vs. multiple), morphology (papillary vs. nonpapillary), tumor grade (low vs. high), staging (Ta vs. T1 vs.T2 at least), and IDO1 expression [negative and weak positive (+) vs. strong positive]; HR = hazard ratio; 95% CI = 95% confidence interval.
[b] Cox proportional hazard ratio.TIL = tumor-infiltrating lymphocytes.

(95% CI), 3.33 (1.48−7.52), $P = 0.004$] and stage [HR (95% CI), 5.17 (2.78−7.59), $P = 0.001$] were significantly associated with DSOS. Tumor IDO1 expression was a borderline significant predictor [HR (95% CI), 1.92 (0.91 −4.03), $P = 0.087$] (Table 2; Fig. 2C). Multivariate analysis showed all of the 3 parameters were independent prognostic predictors for DSOS. A total of 86 bladder tumors were available for measuring tumor-infiltrating CD8+ lymphocyte. Tumors with strong IDO1 expression has significantly lower number of tumor-infiltrating CD8+ lymphocytes than those with null or weak IDO1 expression ($P = 0.026$, unpaired t test; Fig. 2D and E).

3.4. External validation of effect of IDO1 expression on survival

To obtain the external validation, data mining from 4 published human bladder carcinoma datasets GSE32548 ($n = 130$), GSE13507 ($n = 164$), GSE48075 ($n = 74$), and GSE31684 ($n = 93$) were used for survival analysis. The results from these 4 cohorts demonstrated that patients with high IDO1 mRNA expression exhibit shorter disease-free survival than did those with lower IDO1 mRNA expression (all P values < 0.05, log-rank test; Fig. 3).

3.5. IDO1 expression is associated with markers of epithelial-mesenchymal transition (EMT)

To explore whether IDO1 can influence the EMT, we first calculated the association between IDO1 expression and EMT markers using the data mining from these 4 published datasets described as above. The results demonstrated IDO1 expression positively correlated with several mesenchymal markers, including ZEB2 (all 4 datasets, $P < 0.05$), vimentin (all 4 datasets, $P < 0.05$), fibronectin (all 4 datasets, $P < 0.05$), Slug (3 datasets, $P < 0.05$), and MMP-9 (3 datasets, $P < 0.05$). In addition, IDO1 expression positively correlated with 1 epithelial marker Desmoplakin (3 datasets, $P < 0.05$; Table 3).

3.6. IDO1 expression is associated with ZEB2 expression

To confirm the positive association of IDO1 expression with certain EMT markers, we selected the ZEB2 protein for further studies. First, we examined the IDO family expression in several human urothelial cancer cell lines by using quantitative RT-PCR and western blotting analyses (Fig. 4A and B). Among them, BFTC 905 and T24 cells had higher IDO1 expression. We measured the daily changes of 3-HAA concentration in the culture media using
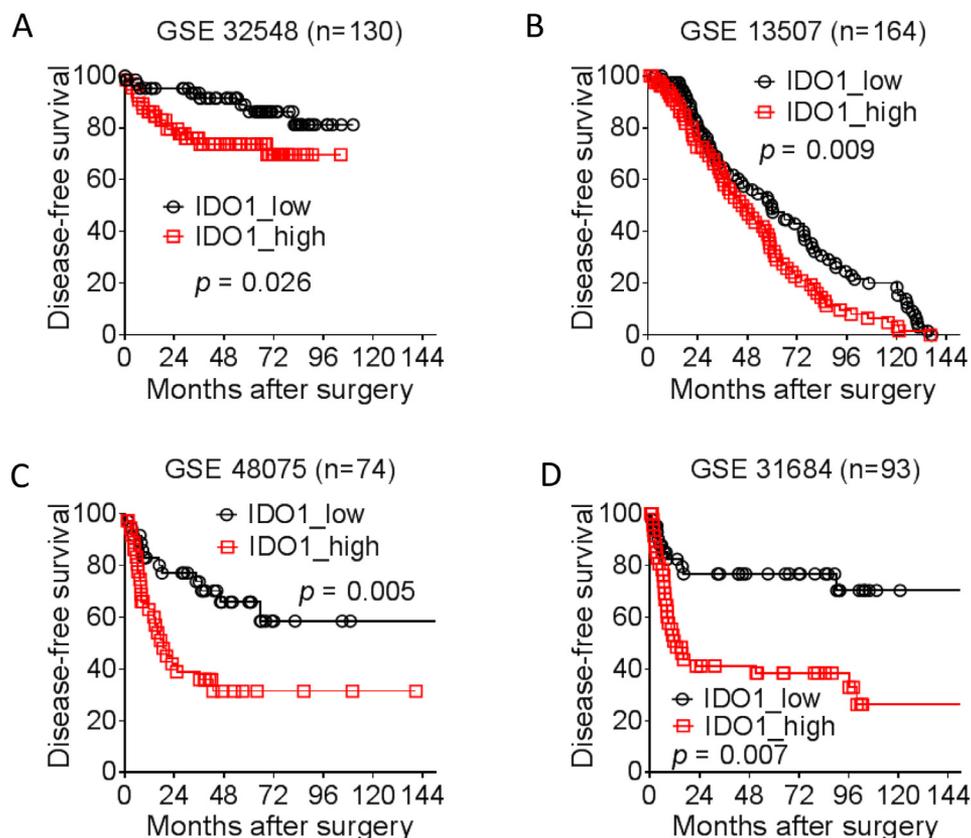

Fig. 3. Survival analysis according to IDO1 expression from 4 published bladder cancer datasets. (A) GSE 32548 ($n = 130$), (B) GSE 13507 ($n = 164$), (C) GSE 48075 ($n = 74$), and (D) GSE 31684 ($n = 93$).

Table 3
The correlation of epithelial-mesenchymal transition markers with IDO1 expression

Markers	GSE32548 (n = 131)			GSE13507 (n = 165)			GSE48075			GSE31684 (n = 93)		
	r*	95% CI	P	r	95% CI	P	r	95% CI	P	r	95% CI	P
Epithelial phenotype												
E-cadherin	−0.119	−0.28, 0.05	0.177	−0.031	−0.18, 0.12	0.697	−0.256	−0.40, −0.09	0.002	−0.014	−0.22, 0.19	0.894
Occludin	0.037	−0.14, 0.21	0.677	0.009	−0.14, 0.16	0.913	0.069	−0.10, 0.23	0.414	−0.249	−0.43, −0.05	0.016
Desmoplakin	0.312	0.15, 0.46	0.0003	0.216	0.07, 0.36	0.005	0.270	0.11, 0.41	0.001	0.136	−0.07, 0.33	0.194
Mesenchymal phenotype												
N-cadherin	0.002	−0.17, 0.17	0.980	0.253	0.10, 0.39	0.001	−0.257	−0.40, −0.10	0.002	0.164	−0.04, 0.36	0.117
Fibronectin	0.409	0.26, 0.54	<0.0001	0.213	0.06, 0.35	0.006	0.177	0.01, 0.33	0.035	0.320	0.12, 0.49	0.002
FOXC2	−0.026	−0.20, 0.15	0.772	0.057	−0.10, 0.21	0.470	0.198	0.03, 0.35	0.018	-	-	-
Goosecoid	−0.081	−0.25, 0.09	0.352	0.055	−0.10, 0.21	0.482	−0.114	−0.27, 0.05	0.177	-	-	-
Rho	0.032	−0.14, 0.20	0.719	−0.155	−0.30, −0.002	0.046	0.072	−0.09, 0.23	0.397	−0.157	−0.35, 0.05	0.133
Snail1 (Snail)	0.099	−0.07, 0.27	0.260	0.324	0.18, 0.45	<0.0001	0.053	−0.11, 0.22	0.526	0.0288	−0.18, 0.23	0.784
Snail2 (Slug)	0.252	0.084, 0.41	0.004	0.249	0.10, 0.39	0.001	0.332	0.18, 0.47	<0.0001	0.168	−0.04, 0.36	0.107
Sox10	0.080	−0.09, 0.25	0.364	0.111	−0.04, 0.26	0.157	−0.034	−0.20, 0.13	0.688	−0.191	−0.38, 0.01	0.066
Twist	0.103	−0.07, 0.27	0.243	0.361	0.22, 0.49	<0.0001	0.095	−0.07, 0.30	0.261	0.192	−0.01 0.38	0.065
Vimentin	0.481	0.34, 0.60	<0.0001	0.316	0.17, 0.45	<0.0001	0.343	0.19, 0.48	<0.0001	0.408	0.22, 0.56	<0.0001
GSK-3βn	0.206	0.04, 0.37	0.018	−0.138	−0.28, 0.02	0.077	0.110	−0.06, 0.27	0.192	-	-	-
ILK	0.062	−0.11, 0.23	0.484	−0.061	−0.21, 0.09	0.434	0.136	−0.03, 0.29	0.108	-	-	-
MMP-2	0.141	−0.03, 0.31	0.108	0.265	0.12, 0.40	0.070	0.203	0.04, 0.36	0.016	0.207	0.004, 0.39	0.046
MMP-3	0.274	0.12, 0.43	0.002	0.304	0.16, 0.44	0.092	0.276	0.12, 0.42	0.0009	0.210	0.006, 0.40	0.043
MMP-9	0.610	0.49, 0.71	<0.0001	0.486	0.36, 0.59	0.236	0.491	0.35, 0.61	<0.0001	0.366	0.18, 0.53	0.0003
β-catenin	−0.171	−0.33, 0.001	0.052	0.100	−0.05, 0.25	0.200	0.206	0.04, 0.36	0.014	−0.136	−0.33, 0.07	0.182
ZEB1	0.092	−0.08, 0.26	0.0002	0.025	−0.13, 0.18	0.755	0.130	−0.04, 0.29	0.124			
ZEB2	0.417	0.26, 0.55	<0.0001	0.200	0.05, 0.34	0.010	0.292	0.13, 0.44	0.0004	0.516	0.35, 0.65	<0.0001

the 3 cell lines (TCCSUP and BFTC905, and BFTC909; Fig. 4C). The results showed the results were consistent with IDO1 mRNA expression (Fig. 4A−C). Further, we also found IDO1 expression is positively associated with metastatic potential in the model of T24 sublines, in which T24-B (derived from bone metastatic site) has the highest IDO1 mRNA and protein expression (Fig. 4D). While knocking-down IDO1 expression in T24-B cells, the ZEB2 expression is also down-regulated, as well as the decreased KYN concentration in the cultured media (Fig. 4E−G). Finally, cell proliferation was also inhibited while knocking-down IDO1 expression (Fig. 4H).

3.7. IDO1 expression up-regulates ZEB2 expression through microRNA signaling

To further explore whether IDO1 can regulate the ZEB2 expression, we measured the change of several ZEB2-regulating microRNA expression. The results demonstrated that miR200c can be up-regulated while knocking down IDO1 expression in T24-B cells, rather than miR-200a, miR-26a or miR-29a (Fig. 5A). Furthermore, with the treatment of miR-200c inhibitor, ZEB2 expression is increased in T24-B cells, as well as decreased E-cadherin expression (Fig. 5B).

4. Discussion

In the current study, we demonstrated that there was a higher frequency of IDO1 expression in the urothelial carcinoma as comparing with the adjacent normal tissue. IDO1

expression is an independent prognostic factor for disease progression and patients' survival, which is associated with decreased number of intratumoral infiltrating CD8+ lymphocyte. Data mining from several published datasets confirmed that patients with higher IDO1 expression exhibit decreased disease-specific survival than those with lower IDO1 expression. Also, IDO1 expression correlated positively with the expression of several EMT markers, including ZEB2, fibronectin, and vimentin. Using the model of T24 sublines, IDO1 expression can up-regulate ZEB2 expression through miR-200c signaling. Taken together, IDO1/miR-200c/ZEB2 signaling may serve as a promising target for bladder cancer therapy. Although IDO1 inhibitor (Epacadostat) plus with anti-PD-1 immunotherapy failed to show any additional efficacy in a phase III trial of melanoma [7], our data provided a hint that IDO1 expression might serve as a predictive marker for IDO1-targeted immunotherapy in urothelial cancer patients.

Higher level of both tryptophan metabolites have been found in the urine of bladder cancer patients when compared with urine of either normal subjects or of those with other malignancies [22]. In the current study, we found patients with urothelial carcinoma have lower urine tryptophan concentration and higher 3-HAA/tryptophan ratio as compared with those urological patients without urothelial carcinoma. The result is consistent with the findings that there is higher IDO1 expression exists in the urothelial tumors than in the benign adjacent tissues. To serve as an ideal urine marker for detecting urothelial carcinoma, it requires more case to clarify the clinical significance.
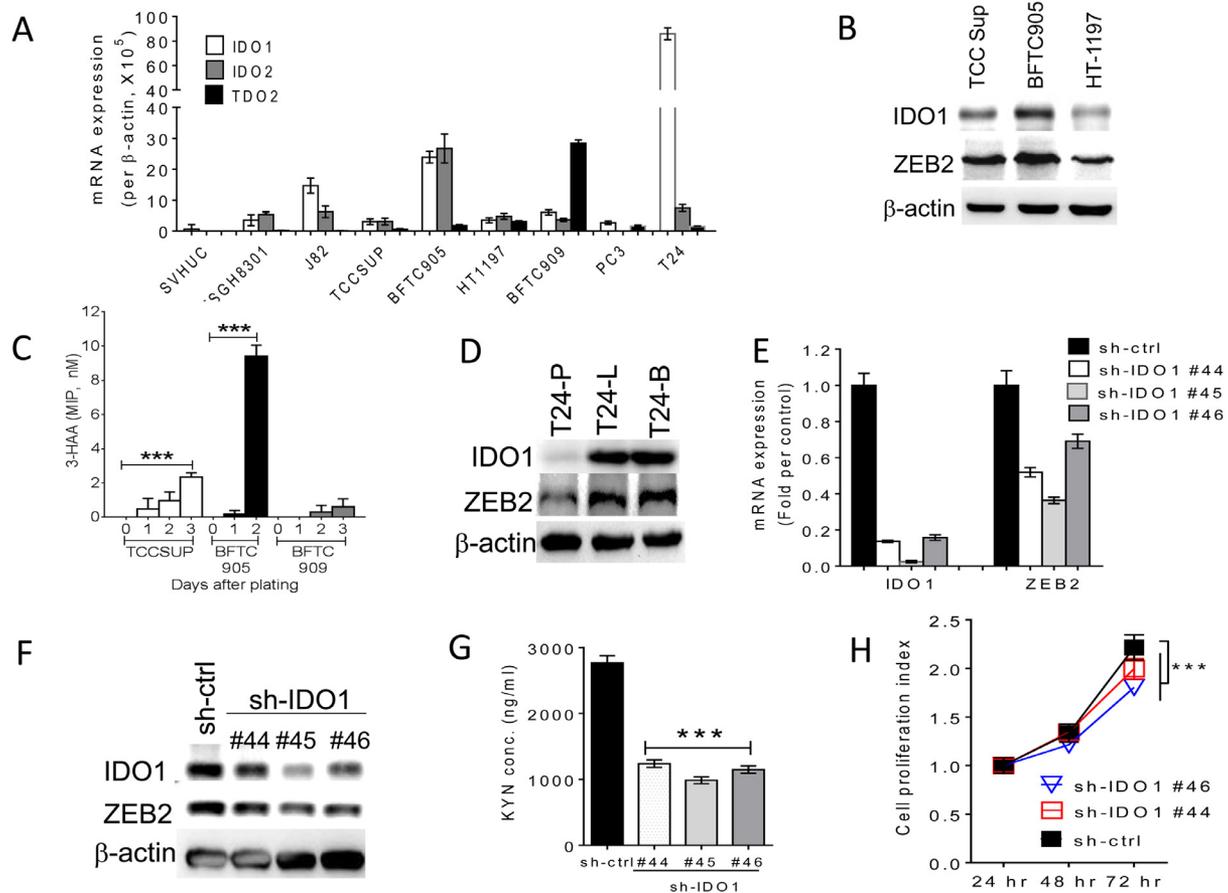
Fig. 4. IDO1 expression is associated with ZEB2 expression. (A) Quantitative RT-PCR of IDO1, IDO2, and TDO2 mRNA in several human bladder cancer cell lines. (B) Western blotting for IDO1 and ZEB2 protein in TCCSUP, BFTC905 and HT-1197 cells. (C) 3-HAA levels in culture media after plating of 3 urothelial carcinoma cells. (D) Western blotting of IDO1 and ZEB2 protein in T24 sublines. (E) Quantitative RT-PCR, and (F) western blotting of IDO1 and ZEB2 mRNA and protein in T24-B cell and its IDO1-knocking-down clones. (G) KYN level of cultured media in T24-B cell and its IDO1-knocking-down clones. (H) MTT assay for cell proliferation in T24-B cells and IDO1-knocking down clones. *** denotes $P < 0.001$.

Many studies reported on the role of IDO1 expression in the immune cells, and only few focus on the role of IDO1 expression in tumor cell. IDO1 expression is increased in thyroid carcinoma and correlates with the presence of Foxp3-positive Treg cells [23]. IDO1 expression in melanoma cell can recruit myeloid-derived suppressor cells and result in Treg-associated immunoresistance [10]. Tumor IDO expression in B-cell lymphoma can inhibit T cells expressing CD19-specific chimeric antigen receptors therapy. Either IDO inhibitors or IDO-depleting agents (such as fludarabine and cyclophosphamide) can restore the efficacy of CD19-specific chimeric antigen receptors therapy [24]. Recent literature revealed that IDO1 expression might be positively or negatively associated with the presence of tumor-infiltrating lymphocyte, which is dependent on either inducible or constitutive IDO1 expression in tumor cells. IDO1 expression can be induced by activated T cell via IFN-γ. Tumors with inducible IDO1 expression is usually belonged to T-cell inflamed, and those with constitutive IDO1 expression is non-T cell-inflamed [25]. There were 33% non-T cell-inflamed, 36% T cell-inflamed, and the

others intermediate based on the data mining analysis of 267 muscle-invasive bladder cancer samples from TCGA database. Also, there was a higher frequency of FGFR3 mutation in non-T cell-inflamed tumors [11]. In our cohort, we found IDO1 expression is up-regulated in urothelial carcinoma and is inversely associated with the number of intratumoral CD8+ lymphocytes. Since more than 80% tumors in the current study were nonmuscle-invasive bladder tumor, it might be the reason why such inverse correlation is conflicted with the finding from TCGA database [11]. Our finding supports that tumor IDO1 expression may drive the development of an intratumoral immunosuppressive microenvironment in human bladder urothelial carcinoma.

Not only cell migration and invasion, ZEB2 can mediate several pathways regulating cell proliferation and apoptosis in glioma cells and tumor ZEB2 expression predicts an unfavorable outcome in human glioma patients [14]. In human bladder cancer, ZEB2 expression was more frequent in infiltrating urothelial carcinoma than in high grade non-invasive urothelial carcinoma [26]. Further, ZEB2 is an
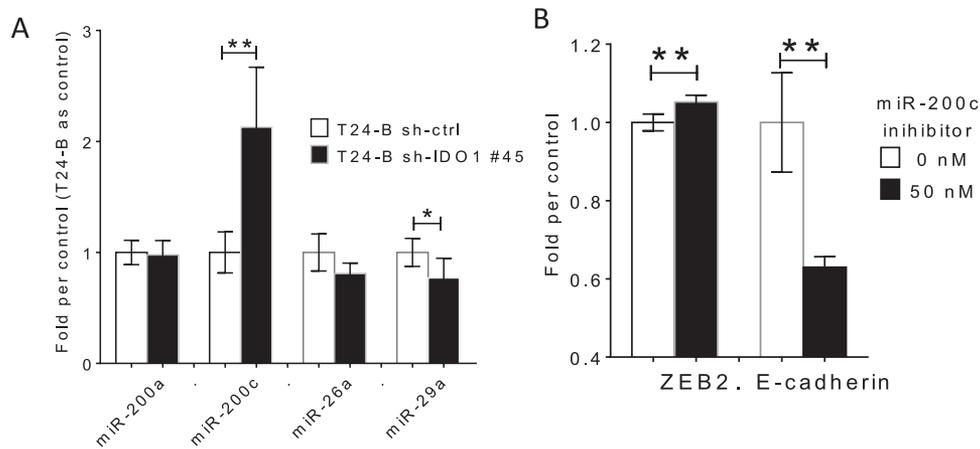
Fig. 5. Increased miR-200c expression in T24-B cell with IDO1-knocking down expression. (A) Quantitative RT-PCR assays for miR-200a, miR-200c, mir-26a, and miR-29a expression in T24-B and IDO1-knocking down cells. (B) ZEB2 and E-cadherin expression in T24-B cells with or without miR-200c inhibitor treatment. * denotes $P < 0.05$; **denotes $P < 0.01$.

independent factor of poor prognosis in a cohort of 72 bladder cancer patients after radiotherapy that can protect cells from DNA-damage induced cell apoptosis [27]. Data mining from several human bladder cancer datasets showed higher tumor IDO1 expression is associated with a worse survival and is associated with the expression of several EMT markers, including ZEB2, fibronectin, and vimentin regulation. Our data validated that IDO1 expression is positively associated with ZEB2 expression and increased cell proliferation in human bladder urothelial cancer cells.

There were several microRNAs targeting and regulating ZEB2 expression [28]. For examples, the miR-200 family can regulate EMT by targeting ZEB1 and ZEB2 in several malignancies [29]. The miR-29a can regulate metastatic properties and epithelial-mesenchymal transition in breast cancer cells through ten-eleven translocation (TET1)/ZEB2 signaling axis [15]. In the current study, we found that IDO1 can up-regulate ZEB through miR-200c signaling.

There were several limitations in the current study. First, it is not easy to collect enough cases for a single-institute survival analysis. The heterogeneity may exist. For example, subcellular location of IDO1 expression may be in the cytoplasm, nucleus or both. Some tumors may present with heterogeneous staining pattern. Although the clinical significance is not known, it may hint that not only enzymatic function but also other biological function IDO1 protein exerts, as shown in the current study. Therefore, we performed external validation from 4 published datasets based on the mRNA level. Second, There are several molecules regulating ZEB2 expression, such as certain microRNAs or TGF-β. It is difficult to control each confounding factor to explore the regulatory mechanisms of ZEB2 expression. Third, it is not easy to understand the biological significance of some IDO1 expression in normal uroepithelia, such as urinary bladder. In the current study, IDO1 staining can be detected in 11 of 26 normal adjacent uroepithelia, including ureter and urinary bladder. Although little information was reported in human, evidence from mice studies

showed IDO1 protein can be expressed in apical epithelial cells of epididymal caput, smooth muscle cells of both the prostate and urinary bladder and capsular cell of the prostate. It is postulated that tryptophan depletion by the presence of IDO1 enzyme plays an important role in diminishing local inflammation by inhibiting the proliferation of some pathogenic bacteria or viruses [30]. Fourth, we used an arbitrary categorical cutoff to group our patients in IHC studies, rather than to use a semiquantitative H score. The results might be barely significant. Since lack of any standard rule for evaluating IDO1 expression from the literature, the obtained result in the current study might be false-positive. Therefore, we used 4 published data to strengthen our result by external validation. Also, the reason that we grouped weakly positive staining tumors with true IDO1 negative tumors is to enhance the purity of IDO1-expressing group and to avoid some equivocal IDO1-expressing tumors.

5. Conclusion

In the current study, we demonstrated that IDO1 expression is increased in the urothelial tumors as compared with normal adjacent urothelial tissues. Tumor IDO1 expression is an independent poor prognostic factor for patients' survival, which is validated with 4 published datasets. Most importantly, IDO1 expression can up-regulate ZEB2 expression though microRNA-200c signaling in human bladder cancer. Such findings may be a promising target for bladder cancer therapy.

Compliance with ethical standards

The approval and institutional oversight of the Institutional Review Board for the Protection of Human Subjects at both of Chia-Yi Christian Hospital (IRB-101014) and National Cheng Kung University Hospital (ER-95-49 and A-ER-102-381) were obtained in the current study.

Y.-S. Tsai et al. / Urologic Oncology: Seminars and Original Investigations 37 (2019) 810.e17–810.e27

Conflict of interest

All authors have no conflict of interest.

Contributions

YS Tsai: Protocol/project development, data analysis, and Manuscript writing/editing.

YC Jou: Data collection, manuscript writing/editing.

HT Tsai: Data collection or management, experimental execution.

IS Cheong: Data collection.

TS Tzai: Protocol/project development.

Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.urolonc.2019.05.005.

References

[1] Antoni S, Ferlay J, Soerjomataram I, Znaor A, Jemal A, Bray F. Bladder cancer incidence and mortality: a global overview and recent trends. Eur Urol 2017;71:96–108.

[2] Gerharz EW, Mansson A, Hunt S, Skinner EC, Mansson W. Quality of life after cystectomy and urinary diversion: an evidence based analysis. J Urol 2005;174:1729–36.

[3] Seisen T, Sun M, Lipsitz SR, Abdollah F, Leow JJ, Menon M, et al. Comparative effectiveness of trimodal therapy versus radical cystectomy for localized muscle-invasive urothelial carcinoma of the bladder. Eur Urol 2017;72:483–7.

[4] Sharma P, Retz M, Siefker-Radtke A, Baron A, Necchi A, Bedke J, et al. Nivolumab in metastatic urothelial carcinoma after platinum therapy (CheckMate 275): a multicentre, single-arm, phase 2 trial. Lancet Oncol 2017;18:312–22.

[5] Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, adaptive, and acquired resistance to cancer immunotherapy. Cell 2017;168:707–23.

[6] Lob S, Konigsrainer A, Rammensee HG, Opelz G, Terness P. Inhibitors of indoleamine-2,3-dioxygenase for cancer therapy: can we see the wood for the trees? Nat Rev Cancer 2009;9:445–52.

[7] Labadie BW, Bao R, Luke JJ. Reimagining IDO pathway inhibition in cancer immunotherapy via downstream focus on the Tryptophan-Kynurenine-Aryl hydrocarbon axis. Clin Cancer Res 2019;25:1462–71.

[8] Fallarino F, Grohmann U, You S, McGrath BC, Cavener DR, Vacca C, et al. Tryptophan catabolism generates autoimmune-preventive regulatory T cells. Transpl Immunol 2006;17:58–60.

[9] Mbongue JC, Nicholas DA, Torrez TW, Kim NS, Firek AF, Langridge WH. The role of Indoleamine 2, 3-Dioxygenase in immune suppression and autoimmunity. Vaccines 2015;3:703–29.

[10] Holmgaard RB, Zamarin D, Li Y, Gasmi B, Munn DH, Allison JP, et al. Tumor-expressed IDO recruits and activates MDSCs in a Treg-dependent manner. Cell Rep 2015;13:412–24.

[11] Sweis RF, Spranger S, Bao R, Paner GP, Stadler WM, Steinberg G, et al. Molecular drivers of the non-T-cell-inflamed tumor microenvironment in urothelial bladder cancer. Cancer Immunol Res 2016;4:563–8.

[12] Lambert AW, Pattabiraman DR, Weinberg RA. Emerging biological principles of metastasis. Cell 2017;168:670–91.

[13] Hill L, Browne G, Tulchinsky E. ZEB/miR-200 feedback loop: at the crossroads of signal transduction in cancer. Int J Cancer 2013;132:745–54.

[14] Qi S, Song Y, Peng Y, Wang H, Long H, Yu X, et al. ZEB2 mediates multiple pathways regulating cell proliferation, migration, invasion, and apoptosis in glioma. PloS One 2012;7:e38842.

[15] Wang H, An X, Yu H, Zhang S, Tang B, Zhang X, et al. MiR-29b/TET1/ZEB2 signaling axis regulates metastatic properties and epithelial-mesenchymal transition in breast cancer cells. Oncotarget 2017;8:102119–33.

[16] Tsai YS, Jou YC, Lee GF, Chen YC, Shiau AL, Tsai HT, et al. Aberrant prothymosin-alpha expression in human bladder cancer. Urology 2009;73:188–92.

[17] Tsai YS, Lai CL, Lai CH, Chang KH, Wu K, Tseng SF, et al. The role of homeostatic regulation between tumor suppressor DAB2IP and oncogenic Skp2 in prostate cancer growth. Oncotarget 2014;5:6425–36.

[18] Kim WJ, Kim EJ, Kim SK, Kim YJ, Ha YS, Jeong P, et al. Predictive value of progression-related gene classifier in primary non-muscle invasive bladder cancer. Mol Cancer 2010;9:3.

[19] Riester M, Taylor JM, Feifer A, Koppie T, Rosenberg JE, Downey RJ, et al. Combination of a novel gene expression signature with a clinical nomogram improves the prediction of survival in high-risk bladder cancer. Clin Cancer Res 2012;18:1323–33.

[20] Lindgren D, Sjodahl G, Lauss M, Staaf J, Chebil G, Lovgren K, et al. Integrated genomic and gene expression profiling identifies two major genomic circuits in urothelial carcinoma. PloS One 2012;7:e38863.

[21] Choi W, Porten S, Kim S, Willis D, Plimack ER, Hoffman-Censits J, et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell 2014;25:152–65.

[22] Boyland E, Williams DC. The estimation of tryptophan metabolites in the urine of patients with cancer of the bladder. Biochem J 1955;60:v.

[23] Moretti S, Menicali E, Voce P, Morelli S, Cantarelli S, Sponziello M, et al. Indoleamine 2,3-dioxygenase 1 (IDO1) is up-regulated in thyroid carcinoma and drives the development of an immunosuppressant tumor microenvironment. J Clin Endocrinol Metab 2014;99:E832–40.

[24] Ninomiya S, Narala N, Huye L, Yagyu S, Savoldo B, Dotti G, et al. Tumor indoleamine 2,3-dioxygenase (IDO) inhibits CD19-CAR T cells and is downregulated by lymphodepleting drugs. Blood 2015;125:3905–16.

[25] van Baren N, Van den Eynde BJ. Tumoral immune resistance mediated by enzymes that degrade tryptophan. Cancer Immunol Res 2015;3:978–85.

[26] Lee H, Jun SY, Lee YS, Lee HJ, Lee WS, Park CS. Expression of miRNAs and ZEB1 and ZEB2 correlates with histopathological grade in papillary urothelial tumors of the urinary bladder. Virchows Arch 2014;464:213–20.

[27] Sayan AE, Griffiths TR, Pal R, Browne GJ, Ruddick A, Yagci T, et al. SIP1 protein protects cells from DNA damage-induced apoptosis and has independent prognostic value in bladder cancer. Proc Natl Acad Sci USA 2009;106:14884–9.

[28] Oba S, Mizutani T, Suzuki E, Nishimatsu H, Takahashi M, Ogawa Y, et al. A useful method of identifying of miRNAs which can down-regulate Zeb-2. BMC Res Notes 2013;6:470.

[29] Korpal M, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J Biol Chem 2008;283:14910–4.

[30] Dai X, Zhu BT. Indoleamine 2,3-dioxygenase tissue distribution and cellular localization in mice: implications for its biological functions. J Histochem Cytochem 2010;58:17–28.