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Original Article

Solute carrier transporters, reduced folate carrier 1 and equilibrative nucleoside transporter 1, as immunohistochemical markers for high-grade malignancy in bladder cancer

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Abstract

Clinicopathological parameters derived from initial transurethral resection of bladder tumor (TUR-Bt) have limitations in predicting tumor progression in bladder cancer. Reduced folate carrier 1 (RFC1) and equilibrative nucleoside transporter 1 (ENT1) are solute carrier (SLC) transporters supporting cellular uptake of endogenous bioactive substances and anti-cancer drugs. The aim of this study was to elucidate the role of SLC transporters in bladder cancer and investigate the potential of RFC1 and ENT1 expression as immunohistochemical markers for high-grade malignancy. We compared T-stage with the immunohistochemical expression of RFC1 and ENT1 and other clinicopathological parameters; moreover, we also used multiple logistic regression model to assess relative contributions for T-stage in bladder cancer (n=130). Concurrently, 57 TUR-Bt-derived imprint cytological samples were stained to evaluate the implication of cytological analysis. Elevated expression levels of RFC1 and ENT1 were significantly correlated with higher T-stage ($p < .0001$) and efficiently predicted tumor progression, compared with other clinicopathological parameters (RFC1, $p = .0325$; ENT1, $p = .0171$). Independent variables of optimal model for predicting T-stage were gender, age, histological grade, expression levels of RFC1 and ENT1. Cytological analysis was consistent with immunostained-tissue data. We reveal RFC1 and ENT1 as potential immunohistocytochemical markers for high-grade malignancy in bladder cancer.

Key words: Reduced folate carrier 1, equilibrative nucleoside transporter 1, solute carrier transporters, urinary bladder neoplasms, immunohistochemistry

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Introduction

Bladder cancer is the seventh and 17th most prevailing cancer in men and women worldwide, respectively. It adds huge economic and social burden on patients[1]. Transurethral resection of bladder tumor (TUR-Bt) is an initial therapeutic strategy for bladder cancer. Approximately 70–90% of bladder cancer is non-invasive on initial diagnosis. However, approximately 60%

patients with non-invasive bladder cancer experience recurrence of which 10–20% cancer turns invasive[2]. Conventionally, clinical and pathological parameters have limitations in predicting tumor progression and prognosis, especially in patients with high-grade non-invasive bladder tumors from initial TUR-Bt[3-4]. Molecular biomarkers that predict clinical outcomes can assist in pathological diagnosis and/ therapeutic strategy. Thus, there is an urgent need to discover ideal biomarkers that can contribute to detecting high malignancy and predicting prognosis for early stage cancer.

Solute carrier (SLC) transporters are a substantial superfamily of transmembrane carriers that play a critical role in cellular uptake of endogenous bioactive substances such as adenosine, xenobiotics, and clinically relevant drugs[5-11].

Solute carrier family 19 member 1 (*SLC19A1*) encodes human reduced folate carrier 1 (RFC1), which is localized to q22.3 on chromosome 21, and its mRNA is expressed in the placenta, liver, and lung[8-10]. RFC1 mediates active intracellular uptake of folates. Within the cells, folic acid is converted to tetrahydrofolate, which is an essential co-factor in *de novo* purine synthesis, DNA synthesis, and methylation of proteins and nucleotides[5-7]. Furthermore, RFC1 transports clinically relevant antifolates such as pemetrexed, raltitrexed, pralatrexate, and methotrexate[7].

Equilibrative nucleoside transporter 1 (ENT1) is encoded by *SLC29A1* which is localized to p21.1-21.2 on chromosome 6 in humans. ENT1 mRNA has been shown to be widely expressed in different cells, tissues, and organs such as erythrocytes, the liver, heart, spleen, kidney, lung, intestine, and brain[8-9]. ENT1 is reported to mediate cellular uptake and excretion of purine and pyrimidine nucleosides, and also play a significant role in the salvage pathway of nucleotide biosynthesis[8-10]. Additionally, ENT1 has been identified as transporter for anti-cancer nucleoside drugs such as cladribine, cytarabine, fludarabine, capecitabine, and gemcitabine[9].

On the other hand, previous literature has demonstrated that SLC transporters are involved in cell proliferation and their expression depends on cell cycle progression and cell differentiation, which is observed to be at peak level during G1 to S transition[5, 10, 11]. Thus, we supposed that high expression levels of SLC transporters can be potential immunohistochemical markers for high-grade malignancy. Many earlier studies have reported that SLC transporters are expressed in many malignant tumors such as pancreas, colorectal, lung, and other cancers, and its over-expression is associated with poor prognosis[12-14]. However, their expression pattern and clinicopathological significance in bladder cancer is still unknown.

The aim of this study was to reveal the characteristic expression of SLC transporters in bladder cancer, and determine whether the expression levels of SLC transporter proteins can be used as immunohistochemical markers for diagnosing high-grade malignancy. At first, we comprehensively analyzed immunohistochemical expression of 12 SLC transporters in bladder cancer cells using tissue array sections. Further, we focused on two SLC transporters, RFC1 and ENT1, and analyzed whether their expressions levels could be useful markers to predict tumor progression. Concurrently, imprinted cytology samples of TUR-Bt were stained to evaluate the possibility of cytological analysis.

Materials and methods

Prescreening of SLC transporters using tissue array

Prior to experimentation, we immunohistochemically analyzed the expression levels of 12 different types of SLC transporters using tissue array sections and confirmed their expression in bladder cancer. Table 1 lists the 12 SLC transporters that were prescreened, information on the primary antibody, and experimental conditions used. We selected the transporters that were involved in the uptake of anti-cancer drugs administered during chemotherapy for bladder cancer or other cancers. Tissue array sections consisted of six bladder cancer samples corresponding to different T-stage and histological grade (Table 2). Additionally, a few samples consisting of non-cancerous epithelium were used as controls.

Patients and samples

We obtained 130 isolated bladder cancer tissues samples before chemotherapy by initial TUR-Bt and 52 non-cancerous tissues by bladder biopsy at Higashiyamato Hospital from January 2013 to December 2014. The study included 105 males and 25 females with an average age of 73.8 years that were diagnosed with bladder cancer. In addition, 40 males and 12 females with an average age of 74 years were enrolled as non-cancerous cases. Of the 130 tissue samples, 57 imprinted cytology samples could be collected, comprising 46 males and 11 females with an average age of 73.2 years.

Tissue samples were fixed with 10% neutral buffered formalin, embedded in paraffin, and sectioned into 4 μm-thick sections. Further, sections were stained with hematoxylin and eosin for pathological diagnosis. Clinical parameters were assigned based on seventh edition of Tumor, Node, Metastasis (TNM) classification of

Table 1. SLC transporters for prescreening and their experimental conditions of immunohistochemical study

Antibody (Gene)	Type of antibody	Dilution	Antigen retrieval	Source	Anti-cancer drugs
RFC1 *(SLC19A1)*	Rabbit polyclonal	1:100	TE9	Atlas antibodies, HPA024802; Stockholm, Sweden	Methotrexate, Pemetrexed etc.
OATP1B1 *(SLC21A6)*	Rabbit polyclonal	1:200	TE9	Abcam, ab15442; Cambridge, UK	Methotrexate, Paclitaxel, etc.
OCT1 *(SLC22A1)*	Rabbit polyclonal	1:500	EDTA	MBL, BMP093; Aichi, Japan	Cisplatin, Imatinib, etc.
OCT2 *(SLC22A2)*	Rabbit polyclonal	1:200	EDTA	Atlas antibodies, HPA008567; Stockholm, Sweden	Cisplatin, Oxaliplatin, etc.
OCT3 *(SLC22A3)*	Rabbit clone EPR6630	1:2000	CB6	Abcam, ab124826; Cambridge, UK	Irinotecan, Oxaliplatin, etc.
OCTN1 *(SLC22A4)*	Mouse polyclonal	1:2000	CB7	Abnova, H0006583-A01; Walnut, CA, USA	Doxorubicin
OAT1 *(SLC22A6)*	Rabbit polyclonal	1:1000	TE9	TransGenic, KE038; Kumamoto, Japan	Cyclophosphamide, Methotrexate, etc.
OAT2 *(SLC22A7)*	Rabbit polyclonal	1:200	TE9	TransGenic, KE031; Kumamoto, Japan	5-Fluorouracil, Methotrexate, etc.
OCT6 *(SLC22A16)*	Rabbit polyclonal	1:400	CB7	Aviva Systems Biology, ARP44073_T100; San Diego, CA, USA	Bleomycin, Doxorubicin
CNT1 *(SLC28A1)*	Goat polyclonal	1:200	TE9	Santa Cruz Biotechnology, sc-48460; Santa Cruz, CA, USA	5'-DFUR, Gemcitabine
ENT1 *(SLC29A1)*	Rabbit polyclonal	1:1000	CB7	Atlas antibodies, HPA012384; Stockholm, Sweden	Cladribine, Gemcitabine, etc.
CTR1 *(SLC31A1)*	Rabbit polyclonal	1:100	Protease	Abnova, PAB14558; Walnut, CA, USA	Carboplatin, Cisplatin

Abbreviations: CB6, citrate buffer (pH 6); CB7, citrate buffer (pH 7); CNT1, concentrative nucleoside transporter 1; CTR1, copper transporter 1; 5'-DFUR, 5'deoxyfluorouridine; ENT1, equilibrative nucleoside transporter 1; OAT1, organic anion transporter 1; OAT2, organic anion transporter 2; OATP1B1, organic anion transporter 1B1; OCT1, organic cation transporter 1; OCT2, organic cation transporter 2; OCT3, organic cation transporter 3; OCTN1, carnitine/organic cation transporter 1; RFC1, reduce folate carrier 1; SLC transporter, solute carrier transporter; TE9, Tris-EDTA buffer (pH 9).

malignant tumors (UICC) and World Health Organization/International Society of Urologic Pathology (WHO/ISUP) system[15-16].

We categorized the cancer cases based on clinicopathological parameters such as T-stage, histological grade, and MIB-1 index. Each case was divided into two groups respectively; age based on the average, T1 or T2 as invasive group (n = 46) and Ta or Tis as non-invasive group (n = 84). Histological grade was assigned as high (n = 47) and low (n = 83) while MIB-1 index was allocated as high (n = 100) and low (n = 30) based on the rate of positive cells. Cut off value of MIB-1 index was 10% according to the literature[17].

Immunohistochemistry

At first, sections were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase in methanol for 30 min. Antigen retrieval was performed using optimal buffers; Tris-EDTA buffer (pH 9) for RFC1 and citrate buffer (pH 7) for ENT1, in a pressure cooker at 120 °C for 10 min. Next, sections were gradually cooled to room temperature (RT, approximately 25 °C) for 30 min. Sections were rinsed in tap water and subsequently immersed in 0.01 mol/L phosphate-buffered saline (PBS, pH 7.2). We used two primary antibodies: RFC1 (1:100, SLC19A1, rabbit polyclonal; Atlas Antibodies, Stockholm, Sweden) and ENT1 (1:1000, SLC29A1, rabbit polyclonal; Atlas Antibodies). Sections were incubated overnight at 4 °C with the respective primary antibodies in a humidified container. After washing in PBS, sections were incubated with secondary antibody: Histofine Simple Stain MAX-PO (Nichirei Biosciences, Tokyo, Japan) for 1 h at RT. Following rinse in PBS, 3,3'-diaminobenzidine (DAB) liquid chromogen substrate (Agilent Technologies, Santa Clara, CA, USA) was added to sections for 5 min. Subsequently, sections were rinsed in tap water, counterstained with hematoxylin, dehydrated through graded alcohol and xylene, and mounted. For negative control, PBS was used instead of primary antibodies.

Staining of Ki-67 (clone MIB-1; Nichirei Biosciences) for evaluating MIB-1 index was performed using BenchMark NX autostainer (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

Immunocytochemistry

Cells from TUR-Bt tissue samples were imprinted onto aminopropyltriethoxysilane-coated slides. Smears were immediately fixed with 95% ethanol for approximately 2 days at RT and stained for immunocytochemical analysis. Heat-induced antigen retrieval for RFC1 and ENT1 was performed using citrate buffer (pH 6). The procedure after antigen retrieval was same as that of immunohistochemistry.

Immunohistocytochemical analysis

At least two researchers analyzed the slides individually, and immunohistochemically evaluated the part used for diagnosing T-stage and histological grade. Tissue section was considered positive if protein expression was observed in the cell membrane or cytoplasm of target cells on immunohistocytochemical staining for each SLC transporter. The expression levels of SLC transporters were evaluated as low (negative to weakly positive) and high (moderate to strongly positive). Evaluating the percentage of cells or area positive for SLC transporters was difficult, as the majority of tumor cells were positively expressed. If different staining intensity was observed in the same slide, areas with suitable representation of the tumor or intensity of the larger area were chosen. Imprinted cytology samples were evaluated for the same cell morphology as a part of tissue specimens that was determined for immunohistochemical staining.

Statistical analysis

Statistical analysis was performed with JMP® 13 (SAS Institute, Cary, NC, USA). We used Fisher's exact test to analyze the correlation between bladder cancer and non-cancerous samples, additionally T-stage and clinicopathological parameters and immunohistochemical expressions of RFC1 and ENT1. We also used multiple logistic regression model to assess relative contributions for T-stage. First, the objective variable was set as T-stage while the independent variables were gender, age, histological grade, MIB-1 index, and staining intensity of RFC1 and ENT1 (full model). In order to determine the most optimal model for predicting T-stage, we generated the four patterns of multiple logistic regression models (1, MIB-1 index; 2, RFC1 and ENT1; 3, RFC1; 4, ENT1) and compared the corrected Akaike's information criterion (AICc) values of all models. Gender, age and histological grade were always added to the independent variables for the clinical perspective. For all analysis, $p < 0.05$ was considered statistically significant.

Ethical considerations

The study was approved by the ethics committee of Higashiyamato Hospital (No.17-009). Written informed consent for comprehensive research use was obtained from all patients.

Results

Prescreening of SLC transporters using tissue array

From 12 SLC transporters investigated, expression of RFC1 and ENT1 was observed to be associated with T-stage or histological grade of bladder cancer (Table 2). Remarkably, the majority of non-cancerous epithelium did not express RFC1 and ENT1 transporters. Remaining SLCs which were negative or not specific for bladder cancer. Consequently, we focused on RFC1 and ENT1 expression, and investigated their role in bladder cancer and non-cancerous tissue samples.

Table 2. Results of prescreening.

	Tissue array sections No.					
	1	2	3	4	5	6
T-stage	Ta	Tis	Ta	Tis	T1	T2
Histological grade	Low	Low	High	High	High	High
RFC1	1+	1+	2+	2+	3+	3+
OATP1B1	-	-	-	-	-	-
OCT1	-	-	-	-	-	-
OCT2	1+	1+	1+	1+	1+	1+
OCT3	1+	-	1+	1+	-	1+
OCTN1	-	-	-	-	-	-
OAT1	-	-	-	-	-	-
OAT2	-	-	-	-	-	-
OCT6	1+	-	-	1+	-	1+
CNT1	-	-	-	-	-	-
ENT1	1+	1+	2+	2+	2+	3+
CTR1	-	-	-	-	-	

-, negative; 1+, weakly positive; 2+, moderately positive; 3+, strongly positive.
Abbreviation: same as table 1.

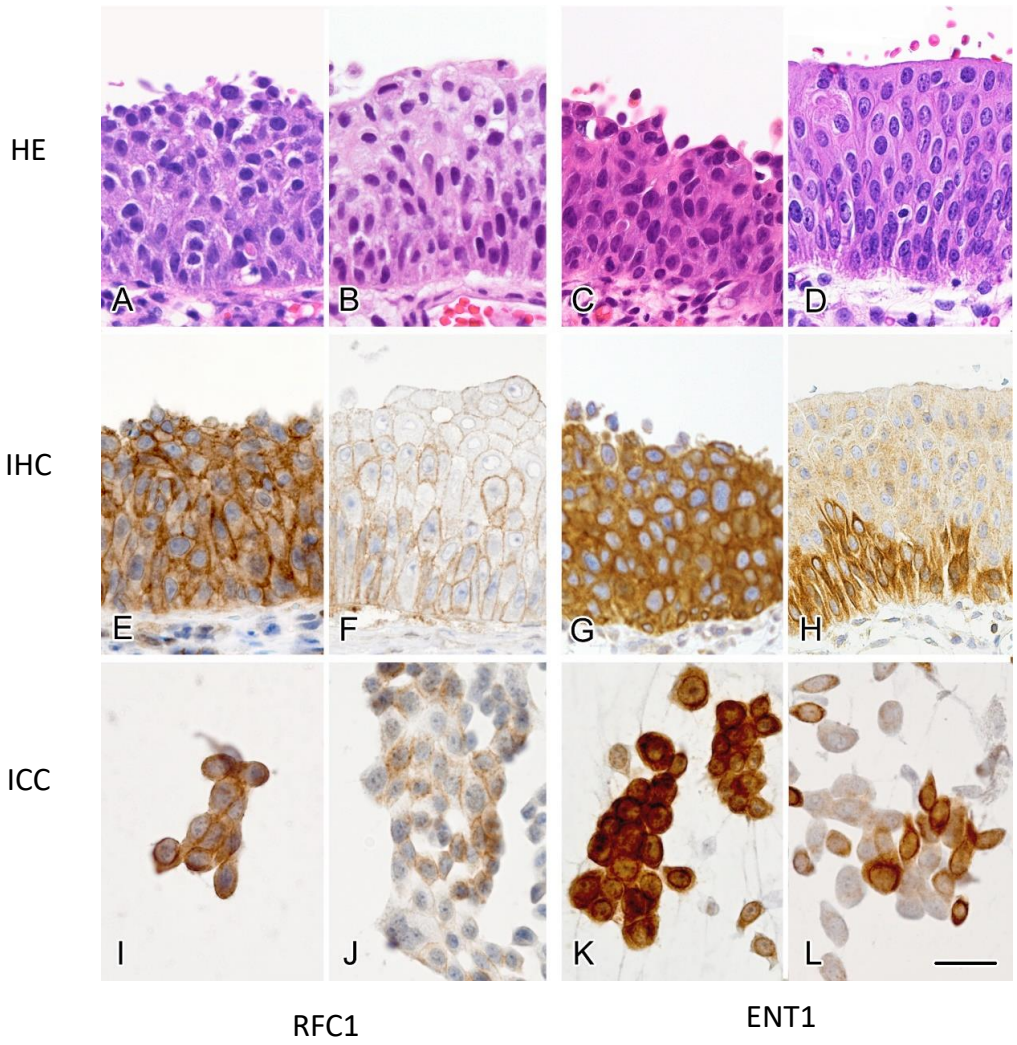

Figure 1. Representative staining pattern of HE staining (A-D), immunohistochemical (E-H) and immunocytochemical expressions (I-L) of RFC1 and ENT1. Three figures in each column represent the same case

A, E, I) High RFC1 expression in cell membrane in bladder cancer. B, F, J) Low RFC1 expression. C, G, K) High ENT1 expression in cytoplasm and partial expression in nuclear membrane in bladder cancer. D, H, L) Low ENT1 expression. Basal pattern observed in the weakly stained tissue and cytology samples. Abbreviations: ENT1, equilibrative nucleoside transporter 1; HE, hematoxylin and eosin; ICC, immunocytochemistry; IHC, immunohistochemistry; RFC, reduced folate carrier. Scale bar=100 μm.

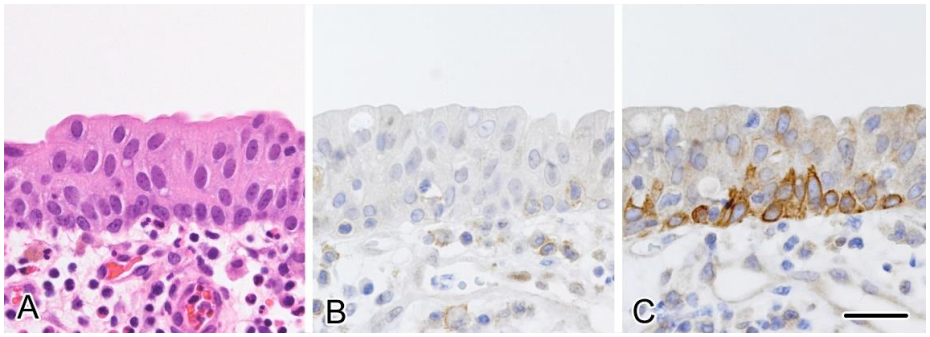

Figure 2. HE staining (A), RFC1 (B) and ENT1 expression (C) in non-cancerous epithelium

B) Majority of non-cancerous epithelium showed negative for RFC1. C) ENT1 is negative or weakly positive for non-cancerous epithelium; however, high expression is observed in basal cells. Scale bar=100 μm.

Characteristics of RFC1 and ENT1 expression

RFC1 expression was found to be localized in the cell membrane (Figs. 1E and 1F) while, ENT1 was predominantly distributed in the cytoplasm and occasionally in the nuclear membrane (Figs. 1G and 1H).

Additionally, we observed following characteristics: (1) ENT1 expression was predominantly localized in the cytoplasm. However, the nuclear membrane tended to be intensely stained compared to the cytoplasm in cases with elevated ENT1 expression (Fig. 1G). (2) In many cases, ENT1 was prominently positive for only basal cells (basal pattern) with weak ENT1 expression whereas cytoplasm of non-basal cells were weakly positive or negative ($p < .0001$) (Table 3, Figs. 1H and 2C). Basal pattern of ENT1 expression was observed in 84.4% of weakly expressed bladder cancer tissues compared to only 21.2% of high ENT1-expressing tissues ($p < .0001$) (Table 3). Further, we evaluated the basal pattern rate based on clinicopathological parameters and found basal pattern in 59.5% of non-invasive group, 72.3% of low histological grade group, and 66.2% of low MIB-1 index group ($p < .0001$, respectively) (Table 3). When ENT1 expression was upregulated, all layers of the tissue were observed to be uniformly stained with no basal pattern. (3) Both the transporters, RFC1 and ENT1, were profoundly expressed in urothelial carcinoma with squamous differentiation ($n = 13$), however, reduced in parts areas of with squamous cells.

Table 3. Basal pattern of ENT1 expression in bladder cancer.

Variables	Number of cases n=130	Basal pattern		OR	95% CI	p-value
		Yes (%) n=56	No (%) n =74			
ENT1 expression						
High	85	18 (21.2)	67 (78.9)	0.05	0.02-0.13	< .0001*
Low	45	38 (84.4)	7 (15.6)			
T-stage†						
Invasive	46	6 (13.0)	40 (87.0)	0.1	0.04-0.44	< .0001*
Non-invasive	84	50 (59.5)	34 (40.5)			
Histological grade						
High	83	22 (26.5)	61 (73.5)	0.14	0.06-0.53	< .0001*
Low	47	34 (72.3)	13 (27.7)			
MIB-1 index‡						
High	62	11 (17.7)	51 (82.3)	0.11	0.05-0.25	< .0001*
Low	68	45 (66.2)	23 (33.8)			

†T-stage was classified as non-invasive stage (Ta and Tis) and invasive stage (≥T1).
‡Cut off value of MIB-1 index was 10%.
* Statistically significant findings.
Abbreviations: CI, confidence interval; ENT1, equilibrative nucleoside transporter 1; OR, odds ratio.

Table 4. Comparison of expression of solute carrier transporters between bladder cancer and non-cancerous epithelium.

	Number of cases n= 182	Expression of RFC1		p-value	Expression of ENT1		p-value
		High (%)	Low (%)		High (%)	Low (%)	
Bladder cancer	130	93 (71.5)	37 (28.5)	< .0001	85 (65.4)	45 (34.6)	< .0001*
Non-cancer	52	2 (3.8)	50† (96.2)		3 (5.8)	49‡ (94.2)	

†Including 28 (53.8%) negative cases.
‡including 12 (23.1%) negative cases.
* Statistically significant findings.
Abbreviation: ENT1, equilibrative nucleoside transporter 1; RFC1, reduced folate carrier 1.

Expression of RFC1 and ENT1 transporters in bladder cancer versus non-cancerous epithelium

As shown in Table 4, RFC1 and ENT1 were abundantly expressed in 71.5% and 65.4% of bladder cancer tissues, respectively (Figs. 1E and 1G). Remaining bladder cancer tissues showed weak expression of RFC1 and ENT1 (Figs. 1F and 1H). On the other hand, majority of non-cancerous epithelium showed negative or weak staining (Figs. 2). Few non-cancerous samples expressed elevated levels of RFC1 and ENT1; however, they were moderately and focally positive. Our results revealed that RFC1 and ENT1 were significantly expressed in cancer cells compared to their expression in non-cancerous epithelium ($p < .0001$, respectively).

Correlation of T-stage with clinicopathological parameters, RFC1 and ENT1

Table 5 illustrates the correlation between T-stage and clinicopathological parameters, immunohistochemical expressions of RFC1 and ENT, and multiple logistic regression analytical data of each variable corresponding to T-stage. Expression levels of RFC1 and ENT1 were significantly enhanced with invasive bladder cancer (97.8% and 95.7%) compared with non-invasive (57.1% and 48.8%) ($p < .0001$, respectively). Even in the non-invasive parts included in the invasive bladder cancers, the expression levels of the two transporters were almost the same as that of the invasive part in many cases. Furthermore, multiple logistic regression analysis showed that RFC1 and ENT1 efficiently predicted tumor progression compared to histological grade or MIB-1 index (RFC1, $p = .0325$; ENT1, $p = .0171$). Alternatively, high histological grade and high MIB-1 index were significantly intense in invasive cases ($p < .0001$ and $p = .0009$); however, they were not predictable factors for T-stage by multiple logistic regression analysis (histological grade, $p = .4599$; MIB-1 index, $p = .5575$). No statistically significant outcomes were observed with gender and age differences.

Table 5. Correlation between T-stage and clinicopathological parameters and expression of RFC1 and ENT1; full model and optimal model of multiple logistic regression analysis for T-stage.

Variables	Number of cases	T-stage†		Fisher's exact test			Full model of multiple logistic regression analysis for T-stage					Optimal model of multiple logistic regression analysis for T-stage				
		Invasive	Non-invasive	OR	95% CI	p-value	β	SE	OR	95% CI	p-value	β	SE	OR	95% CI	p-value
	n=130	n=46	n=84													
Gender																
Male	105	33 (71.7)	72 (85.7)	0.42	0.18-1.01	.0645	0.65	0.55	1.92	0.66-5.98	.2401	0.66	0.55	1.94	0.67-6.02	.2285
Female	25	13 (28.3)	12 (14.3)													
Age																
≥74	71	30 (65.2)	41 (48.8)	2.00	0.94-4.10	.0972	0.55	0.47	1.73	0.68-4.44	.2478	0.59	0.47	1.80	0.72-4.58	.2103
<74	59	16 (34.8)	43 (51.2)													
Histological grade																
High	83	42 (91.3)	41 (48.8)	11.01	3.76-31.97	< .0001*	0.55	0.75	1.74	0.40-8.09	.4599	0.67	0.75	1.96	0.48-8.71	.3504
Low	47	4 (8.7)	43 (51.2)													
MIB-1 index‡																
High	100	43 (93.5)	57 (67.9)	6.79	1.93-23.86	.0009*	0.47	0.81	1.61	0.34-8.88	.5575	N/A	N/A	N/A	N/A	N/A
Low	30	3 (6.5)	27 (32.1)													
RFC1																
High	93	45 (97.8)	48 (57.1)	33.75	5.59-200.85	< .0001*	2.50	1.17	12.20	1.67-253.70	.0325*	2.55	1.16	12.82	1.79-264.68	.0282*
Low	37	1 (2.2)	36 (42.9)													
ENT1																
High	85	44 (95.7)	41 (48.8)	23.07	5.77-91.07	< .0001*	1.99	0.83	7.30	1.69-51.44	.0171*	1.95	0.83	7.04	1.63-49.08	.0182*
Low	45	2 (4.3)	43 (51.2)													
AICc									133.6					131.7		

†T-stage was classified as non-invasive stage (Ta and Tis) and invasive stage (≥T1).
‡Cut off value of MIB-1 index was 10%.
* Statistically significant findings.
Abbreviations: AICc, corrected Akaike's information criterion; β, partial regression coefficient; CI, confidence interval; ENT1, equilibrative nucleoside transporter 1; N/A, not applicable; OR, odds ratio; RFC, reduced folate carrier; SE, standard error.

Table 6. Correlation between expression of solute carrier transporter and clinicopathological parameters.

Variables	Number of cases	RFC1 expression		P value	ENT1 expression		p-value
		High (%)	Low (%)		High (%)	Low (%)	
	n=130	n=93	n=37		n=85	n=45	
Gender							
Male	105	74 (70.5)	31 (29.5)	.8055	63 (60.0)	42 (40.0)	.0093*
Female	25	19 (76.0)	6 (24.0)		22 (88.0)	3 (12.0)	
Age (year)							
≥74	61	43 (46.2)	18 (48.6)	.8471	45 (52.9)	16 (35.6)	.0668
<74	69	50 (53.8)	19 (51.4)		40 (47.1)	29 (64.4)	
T-stage†							
Invasive	46	45 (97.8)	1 (2.2)	< .0001*	44 (95.7)	2 (4.3)	< .0001*
Non-invasive	84	48 (57.1)	36 (42.9)		41 (48.8)	43 (51.2)	
Histological grade							
High	83	77 (92.8)	6 (7.2)	< .0001*	71 (85.5)	12 (14.5)	< .0001*
Low	47	16 (34.0)	31 (65.0)		14 (29.8)	33 (70.2)	
MIB-1 index‡							
High	100	85 (85.0)	15 (15.0)	< .0001*	75 (88.2)	25 (55.6)	< .0001*
Low	30	8 (26.7)	22 (73.3)		10 (11.8)	20 (44.4)	
RFC1 expression							
High	93	N/A	N/A	N/A	76 (81.7)	17 (18.3)	< .0001*
Low	37	N/A	N/A		9 (24.3)	28 (75.7)	

†T-stage was classified as non-invasive stage (Ta and Tis) and invasive stage (≥T1).
‡Cut off value of MIB-1 index was 10%.
* Statistically significant findings.
Abbreviations: ENT1, equilibrative nucleoside transporter 1; N/A, not applicable; RFC, reduced folate carrier.

In addition, when comparing AICc values between multiple logistic regression models, it was shown that the full model was 133.6, pattern 1 was 146.8, pattern 2 was 131.7, pattern 3 was 136.8 and pattern 4 was 136.4. Thus, pattern 2 was the most optimal model. MIB-1 index did not improve prediction accuracy of T-stage, and both RFC1 and ENT1 were necessary for optimal model.

Shown in Table 6, elevated expression levels of RFC1 and ENT1 were also significantly correlated with high histological grade and high MIB-1 index ($p < .0001$, respectively). Moreover, statistically significant correlation was observed between the expression levels of RFC1 and ENT1 ($p < .0001$).

Immunocytochemistry

Seventy-five cytological samples (57.7%) from different areas of tissue were collected for immunostaining of RFC1 and ENT1 expression analysis. Furthermore, 57 imprinted samples (43.8%) were successfully evaluated by immunocytochemistry. Remaining samples were excluded as they did not include any cancer cells or had poor stainability. High expression levels of RFC1 and ENT1 were observed in 80.7% and 68.4% respectively. Expression levels in cytological samples were found to be completely consistent with immunohistochemical data. Though few samples showed minor differences in the staining intensity, we did not observe significant diagnostic differences between tissue and cytological sample analysis (Fig. 1I-L).

Discussion

We found several characteristics following comprehensive analysis of RFC1 and ENT1 staining patterns in bladder cancer tissues. (1) ENT1 expression is predominantly localized in the cytoplasm; however, the nuclear membrane tends to be intensely stained compared to the cytoplasm in cases with high ENT1 expression. ENT1 was found to be primarily expressed in the plasma and mitochondrial membranes and is required for nucleotide synthesis[10]. Mani RS et al. reported that ENT1 is functionally associated with the nuclear membrane and endoplasmic reticulum. The endoplasmic reticulum consists of many different enzymes for nucleotide metabolism and ENT1 might be playing a role in translocation of nucleosides between the cytosolic and luminal compartments[18]. Nuclear envelope-associated ENT1 might be serving as a reserve pool for translocation to the plasma membrane when additional transport capability is needed, such as in excessive proliferation. (2) ENT1 consistently showed basal pattern in many weakly stained tissues. Chan KS proposed that the basal layer in non-invasive bladder cancer has tumorigenic potential[19]. ENT1-positive cell populations in the basal layer might be related to cancer stem cells with urothelial carcinoma. (3) Furthermore, expression levels of both the transporters were elevated in urothelial carcinoma with squamous differentiation. Minato et al. reported that the survival outcomes were different between histological variants and urothelial carcinoma groups in muscle-invasive bladder cancer. They suggested that presence of squamous differentiation predicts poor oncological outcome[20]. Thus, bladder cancer with squamous differentiation might have robust expression of transporters because of high malignant potential. However, parts of squamous epithelium appeared to be poorly stained to these transporters.

We demonstrated that an increase in expression of RFC1 and ENT1 in bladder cancer can distinguish between cancer and non-cancer, and also be associated with cancer progression or malignancy. Histological grade is one of the conventional markers used in determining the malignant potential of bladder cancer[15, 16]. Furthermore, MIB-1 index, which indicates proliferation ability, is shown to have a positive correlation with malignancy in many malignant tumors[17, 21]. However, in this study, multiple logistic regression analysis showed that the expression levels of RFC1 and ENT1 were significant independent predictors compared with histological grade or MIB-1 index in determining tumor progression. Cancer cells have higher proliferative ability to show substantially different expression profiles of SLC transporters as compared to those shown by normal healthy cells. This may be due to upregulation of specific SLCs in tumor cells owing to increased demand for energy and nutritional needs. Since both RFC1 and ENT1 are involving in DNA biosynthesis, it is comprehensible that their high expression levels associated with aggressive progression in bladder cancer. Their expression levels may more directly reflect the cell proliferation in comparison with conventional markers. Moreover, as the two transporters were also strongly expressed in the non-invasive parts of high-expressed invasive cancers, they could be predictable makers for non-invasive cancers. Thus, expression level of RFC1 and ENT1 can aid in distinguishing bladder cancer and estimating the tumor progression in ambiguous cases during pathological diagnosis.

On the other hand, methotrexate-vinblastin-adriamycin-cisplatin (M-VAC) and gemcitabine-cisplatin (GC) therapy are conventional regimens for treating urothelial bladder cancer. RFC1 and ENT1 expression has been shown to facilitate cellular uptake of methotrexate and gemcitabine, respectively, during M-VAC and GC

treatments[6-10, 22]. Unfortunately, the correlation between expression levels of these two transporters and effects of chemotherapy could not be anticipated in this study. However, RFC1 and ENT1 expression in cancer cells has been reported as a key determinant for therapeutic effect or prognosis in many tumors[12-14]. Fleischmann A et al. have described how favorable chemotherapy response was associated with high proliferation of cancer cells in the initial chemotherapy-naive bladder cancer[23]. As RFC1 and ENT1 participate in cellular uptake of anti-cancer drugs as well as cell proliferation, their expression levels can explain the correlation between proliferative ability and effect of chemotherapy. Therefore, RFC1 and ENT1 can also be used as therapeutic targets in bladder cancer.

Such interpretation of tumor progression or malignancy from initial TUR-Bt samples will aid in determining therapeutic strategy during early stages of cancer and ultimately, improve the clinical outcome of patients.

Furthermore, we showed that staining patterns of RFC1 and ENT1 in cytological samples were consistent with that observed in tissue samples. To our knowledge, no other study has yet confirmed the expression of SLC transporter proteins in cytological specimens, especially ENT1 and RFC1. Accomplishments with immunocytochemical analysis suggests the potentiality of immunocytochemical research on transporter proteins using urine cytological specimens. Moreover, our study will promote comprehensive investigations on the role of transporter proteins in other organs using immunocytochemical analysis.

In conclusion, our study revealed that expression of RFC1 and ENT1 is significantly associated with T-stage, and efficiently predicted tumor progression compared with histological grade and MIB-1 index. We conclude that RFC1 and ENT1 may be potential immunohistocytochemical markers for high-grade malignancy in bladder cancer. In future, we expect to replicate our results with analyses of patients with bladder cancer that have undergone chemotherapy.

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