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RESEARCH ARTICLE

Quantitation of rare circulating tumor cells by folate receptor α ligand-targeted PCR in bladder transitional cell carcinoma and its potential diagnostic significance

Fuming Qi · Yuchen Liu · Rongchang Zhao · Xiangjun Zou · Lei Zhang · Jiaqiang Li · Yongqiang Wang · Feiyang Li · Xiaowen Zou · Ye Xia · Xuliang Wang · Li Xing · Cailing Li · Jingxiao Lu · Junlong Tang · Fangjian Zhou · Chunxiao Liu · Yaoting Gui · Zhiming Cai · Xiaojuan Sun

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Abstract Numerous attempts for detection of circulating tumor cells (CTC) have been made to develop reliable assays for early diagnosis of cancers. In this study, we validated the application of folate receptor α (FRα) as the tumor marker to detect CTC through tumor-specific ligand PCR (LT-PCR) and assessed its utility for diagnosis of bladder transitional cell carcinoma (TCC). Immunohistochemistry for FRα was performed on ten bladder TCC tissues. Enzyme-linked immunosorbent assay (ELISA) for FRα was performed on both urine and serum specimens from bladder TCC patients (n=64 and n=20, respectively) and healthy volunteers (n=20 and n=23, respectively). Western blot analysis and qRT-PCR were performed to confirm the expression of FRα in bladder TCC cells. CTC values in 3-mL peripheral blood were measured in 57 bladder TCC patients, 48 healthy volunteers, and 15 subjects with benign urologic pathologies by the folate receptor α ligand-targeted PCR. We found that FRα protein was overexpressed in both bladder TCC cells and tissues. The levels of FRα mRNA were also much higher in bladder cancer cell lines 5637 and SW780 than those of leukocyte. Values of FRα were higher in both serum and urine specimens of bladder TCC patients than those of control. CTC values were also higher in 3-mL peripheral blood of bladder TCC patients than those of control (median 26.5 Cu/3 mL vs 14.0 Cu/3 mL). Area under the receiver operating characteristic (ROC) curve for bladder TCC detection was 0.819, 95 % CI (0.738–0.883). At the cutoff value of 15.43 Cu/3 mL, the sensitivity and the specificity for detecting bladder cancer

Fuming Qi and Yuchen Liu contributed equally to this work.

F. Qi · Y. Liu · X. Zou · Z. Cai
Department of Urological Surgery, Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University, Shenzhen, China

F. Qi · Y. Liu · J. Li · Y. Xia · X. Wang · C. Li · Y. Gui
Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics, Institute of Urology, Peking University Shenzhen Hospital, Shenzhen PKU-HKUST Medical Center, Shenzhen, China

F. Qi
Shantou University Medical College, Shantou, China

Y. Liu · R. Zhao · X. Zou · L. Zhang · J. Li · Y. Wang · F. Li · X. Zou · Y. Xia · X. Wang · L. Xing · Z. Cai (✉)
Shenzhen Key Laboratory of Genitourinary Tumor, Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University, Shenzhen 518035, China
e-mail: caizhiming2000@163.com

F. Zhou
Department of Urology, Sun Yat-Sen University Cancer Center, Guangzhou, China

L. Zhang · L. Xing · C. Liu
Department of Urology, Zhujiang Hospital of Southern Medical University, Guangzhou, China

R. Zhao
Department of Research and Development, GenoSaber Biotech Co., Ltd., Shanghai, China

J. Lu · J. Tang · X. Sun (✉)
Shenzhen Tumor Immuno-gene Therapy Cliniical Application Engineering Lab, Biobank of Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University, 802 Room, Yinhua Building, 3002 West Sungang Road, Futian District, Shenzhen 518035, China
e-mail: xiaojuan26@gmail.com

are 82.14 and 61.9 %, respectively. We concluded that quantitation of CTCs through FRα ligand-PCR could be a promising method for noninvasive diagnosis of bladder TCC.

Keywords Bladder transitional cell carcinoma · Tumor specific ligand-PCR · Circulating tumor cell · Folate receptor α

Introduction

Bladder transitional cell carcinoma (TCC) is an important cause of morbidity and mortality, and about 72,570 new cases and 15,210 deaths are estimated to have occurred each year worldwide [1, 2]. Generally, the higher cancer grade indicates worse prognosis. In patients with low-grade Ta disease, the 15-year progression-free survival rate is 95 %. In patients with high-grade Ta tumors, the 15-year progression-free survival rate is 61 %, and the disease-specific survival rate is 74 % [3]. Ten to twenty percent of the superficial tumors (stages Ta, T1, or tumors in situ [Tis]) will progress to muscle-invasive disease (T2–4) [4]. Hence, early diagnosis of bladder TCC could help improve the prognosis of patients.

As potential biomarkers of bladder cancer, p53, pRB, p21, p27, and cyclin E1 have been shown to be associated with poor oncologic outcomes and disease recurrence. However, there are no clinically usable molecular markers that can guide us in early diagnosis [5–7]. Moreover, multifocal urothelial carcinomas are frequently monoclonal, whereas others show oligoclonality. The presence of oligoclonality has implications for the potential efficacy of novel molecular biomarkers for bladder cancer [8, 9].

Recent molecular and clinical studies have shown that invasion may occur very early in tumor development, and circulating tumor cells (CTC) are released into circulation in an early phase of cancer disease. Many research have emphasized the potential importance of specific and sensitive detection of circulating tumor cells [10–12]. For several decades, numerous attempts have been made to develop reliable assays to detect these rare cells. However, much of them turned out to be rather cumbersome and inefficient. Furthermore, the specificity, sensitivity, and reproducibility of these technologies still need to be improved [11].

In this research, we explore an effective strategy for enrichment, characterization, and quantification of CTC based on the high expression of folate receptor α (FRα) in bladder TCC and validate its diagnostic significance. To the best of our knowledge, this is the first study reporting that FRα can be utilized as a tumor marker for detecting CTC in bladder TCC.

Materials and methods

Patient characteristics

Fifty-seven bladder TCC patients in the study were enrolled from Zhujiang Hospital of Southern Medical University and Sun Yat-Sen University Cancer Center between October 2012 and September 2013. Bladder transitional cell carcinoma was diagnosed histopathologically. All subjects had either localized, muscle-invasive disease or metastatic urothelial cancer. Evaluation of 48 healthy volunteers included a complete history and physical examination and imaging studies to exclude other forms of malignancy in these individuals. Samples were also collected from 15 subjects with benign urologic pathologies including urinary tract infection, lithangiuria, prostatitis, and prostatic hypertrophy. All patients and healthy people who voluntarily enrolled in the study signed an informed consent and were properly informed before recruitment for the study, according to the regulations of the institutional ethics review boards. The patient characteristics are given in Table 1.

Folate receptor alpha expression validation

RT-qPCR and western blot analysis

The leukocyte was collected from the 15-mL blood of each health volunteer after schizolysing red blood cells. The human bladder TCC cell line 5637 (ATCC® HTB-9™) and SW780 (ATCC® CRL-2169™) maintained in our institute were used to identify antibody for folate receptor alpha.

The protein was extracted by Pierce® BCA Protein Assay Kit (Thermo, UT, USA) according to the company's protocol. Western blot analysis was performed as described previously [13] using the secondary antibody anti-folate receptor 1 (adult) (FOLR1) (Sigma-Aldrich, MO, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control.

RNA was extracted by the RNeasy Mini Kit according to company's protocol (Qiagen, Hilden, Germany). Reverse transcription of total RNA and qPCR reactions were performed as described previously [14] using the protocol of PrimeScript RT reagent kit (Takara, Japan). The primers for FRα were 5′-CCCCAGGACTAGTTGCATGA-3′ (forward) and 5′-TCCACGGTGGTTCCAGTTGAATCTA-3′ (reverse). The primers for GAPDH were 5′-AGCAAGAGCACAAG AGGAAGAG-3′ (forward) and 5′-TCTACATGGCAACT GTGAGGAG-3′ (reverse).

Sandwich-type enzyme-linked immunosorbent assay

The FRα protein values in the serum and urine of the bladder TCC patients and healthy volunteers were determined by

Table 1 CTC values in patients and healthy volunteers

Characteristic	Subjects			CTC value (Cu/3 mL)		
	No.	%	Median	Range	95 % CI	*p* Value
Noncancer subjects	63	100	14.0	1.8–24.36	12.5–15.6	<0.001[a]
Bladder TCC cases	57	100	26.5	7.0–105.4	18.6–31.4	
Gender distribution						
Male	51	89.5	26.7	7–105.4	18.8–31.8	
Female	6	10.5	19.9	15.6–81.5	15.6–81.5	0.658[a]
Pre/post operation						
Pre	20	35.1	26.5	10.3–105	16.5–37.2	
Post	37	64.9	26.5	7–83.9	18.3–31.8	0.699[a]
Incipient or recurrent						
Recurrent	20	35.1	32.8	9.0–105.4	16.1–49.0	
Incipient	37	64.9	22.0	7.0–64.6	18.1–29.0	0.134[a]
Pathology TNM stage						
Ta	13	22.8	20.2	10.7–105.4	15.6–37.2	
T1	16	28.1	28.4	9.0–81.5	13.2–53.4	
≥T2	21	37	30.3	7.0–87.8	18.8–36.5	
Lymph or distant metastasis	7	6.7	16.5	9.5–31.4	9.5–31.4	0.335[b]
Tumor grade						
PUNIMP	1	1.8	22.2			
HGPUC	31	54.4	28.4	9.0–87.8	18.3–35.2	
LGPUC	25	43.9	21.4	7.0–105.4	15.7–31.8	0.575[a]

CTC circulating tumor cells, *CI* confidence interval, *TCC* transitional cell carcinoma, *PUNIMP* papillary urothelial neoplasm of low malignant potential, *HGPUC* high-grade papillary urothelial carcinoma, *LGPUC* low-grade papillary urothelial carcinoma

[a] Mann–Whitney *U* test

[b] Kruskal–Wallis nonparametric analyses of variance

sandwich-type enzyme-linked immunosorbent assays (ELISA) for folate receptor 1 adult (FOLR1) according to the manufacturer's instructions (Uscn Life Science Inc., Wuhan, China). Samples were assayed in duplicate with all values calculated as the mean of the two measurements. The absorbance was read at 450 nm in a 96-well microtiter plate reader.

Immunohistochemical analysis of the expression of folate receptor alpha protein

An immunohistochemical assay was performed to examine FRα expression in the tissue samples. All procedures were performed as previously published [15]. The FRα protein was detected by using an anti-folate binding protein antibody ab137347 (Abcam; Cambridge, MA, USA). The tissue specimens were incubated overnight at 4 °C with anti-folate antibody (1:600). The negative control for immunohistochemical analysis was obtained by replacing the primary antibodies with an antibody diluent. After being washed in phosphate-buffered saline (PBS), the sections were treated with MaxVision™ UltraSensitive™ SP (rabbit) IHC Kit (Maixin Bio, Fujian, China) at 37 °C for 15–20 min. The tissue sections were immersed in 3-amino-9-ethyl carbazole, counterstained with Mayer's hematoxylin, dehydrated, and finally mounted in crystal mount. The formalin-fixed, paraffin-embedded sections were reviewed for the degree of immunostaining using the method as described in the previous work [15].

CTC detection

CTC enrichment

Blood sample (3 mL) was collected in EDTA tubes and immediately transferred in low-temperature condition to our laboratory within 5 h of venipuncture and then was processed to prepare for PCR analysis. CTCs were enriched by lysis of erythrocytes and subsequent depletion of leukocytes referring to the manufacturer's protocol (provided by GenoSaber Biotech Co., Ltd., Shanghai, China). Briefly, the anticoagulant whole blood samples were first lysed by red blood cell lysis buffer (*v*/*v*, 1:4) for 15 min on ice. The cells were then treated with 200-μL anti-CD45-coated magnetic beads for 30 min to deplete leucocytes. After that, the enriched CTC was incubated with 10-μL labeling buffer and labeled with a conjugate of a tumor-specific ligand folic acid and a synthesized oligonucleotide for 40 min at room temperature. The cells were then washed three times with 1-mL wash buffer at 500 g to remove the unbound conjugates. Finally, the cells were treated with 120-μL stripping buffer to remove the ligand-oligonucleotide conjugates. The supernatant were collected by centrifugation and neutralized by 24-μL neutralization buffer for further PCR analysis. After that, the extended conjugate was

amplified and analyzed using a TaqMan probe based on quantitative PCR method. In the above method, the circulating tumor cells were identified as folate-receptor-positive cells when they were labeled by folate-linked oligonucleotides.

CTC quantitation

The detection and quantification of component includes PCR reaction buffer, primers, deionized water, positive and negative cell controls, and PCR controls and standards. The primer sequences were listed as follows. First, the RT primer to extend the striped FRα ligand is 5′-CTCAACTGGTGTCG TGGAGTCGGCAATTCAGTTGAGGGTTCTAA-3′; then, the following forward primer, 5′-TATGATTATGAGGCAT GA-3′, and reverse primer, 5′-GGTGTCGTGGAGTCG-3′, were used to amplify the extended FRα ligand; TaqMan probe is 5′-FAM-CAGTTGAGGGTTC-MGB-3′. Real-time quantitative polymerase chain reaction was performed using the tumor-specific ligand PCR on ABI ViiA7 real-time PCR (Life Technologies, USA). Two and a half microliters of the prepared samples was added into a 25-μL PCR reaction system following the manufacturer's instruction manual. The PCR reaction conditions were as follows: denaturation at 95 °C for 2 min, annealing at 40 °C for 30 s, extension at 72 °C for 30 s, then cooling at 8 °C for 5 min; 40 cycles of denaturation at 95 °C for 10 s, annealing at 35 °C for 30 s, and extension at 72 °C for 10 s. A serial of standards containing oligonucleotides (10–14 to 10–9 M, corresponding to 2 to 2×10^5 CTC units/3 mL blood) is used for CTC quantification. All patient samples were tested in duplicates with six standards and three quality controls. The observed CTC counts were computed as previously described [16].

Clinical evaluations and statistical analyses

To evaluate the clinical utility, we conducted a double-blind study on patients with benign and malignant bladder diseases. In order to evaluate the difference among test groups, we used the Mann–Whitney *U* test and Kruskal–Wallis nonparametric analyses (SPSS 17.0). To determine the threshold, specificity, and sensitivity of the method, the data from patients and healthy volunteers were subjected to the nonparametric receiver operating characteristic (ROC) analysis by SPSS 17.0.

Results

Overexpression of folate receptor alpha in TCC

Overexpression of folate receptor alpha in TCC cell line

To determine the expression level of FRα in the bladder TCC cells, Western blot analysis was performed. The results showed that FRα protein is highly expressed in the bladder TCC cells (Fig. 1a). We further validated the expression level of FRα messenger RNA (mRNA) in the bladder TCC cells by qRT-PCR analysis. Approximately 650-fold and 15-fold increments were observed when compared to the leukocyte collected from the bladder TCC patients (Fig. 1b).

FRα protein value determined by enzyme-linked immunosorbent assay

Totally, as shown in Fig. 1c, 43 serum samples including 20 of the bladder TCC patients and 23 of the healthy volunteers were tested by ELISA, and the results showed significant difference ($p=0.0078$). Similarly, urine samples of 64 bladder TCC patients and 20 healthy volunteers were analyzed (Fig. 1d); ELISA assay indicated that the FRα protein value in the urine is much higher in the bladder TCC patients than that in healthy volunteers ($p=0.0001$).

Immunohistochemical analysis of the expression of FRα protein in ten paraffin-embedded bladder TCC tissue samples

Ten paraffin-embedded bladder cancer (BCa) tissues and their paired specimens of adjacent normal tissues were determined by immunohistochemical analysis. The expression of FRα protein in the ten tumor tissue samples was higher than that in the adjacent normal tissue samples. FRα protein was mainly localized at cytoplasm of

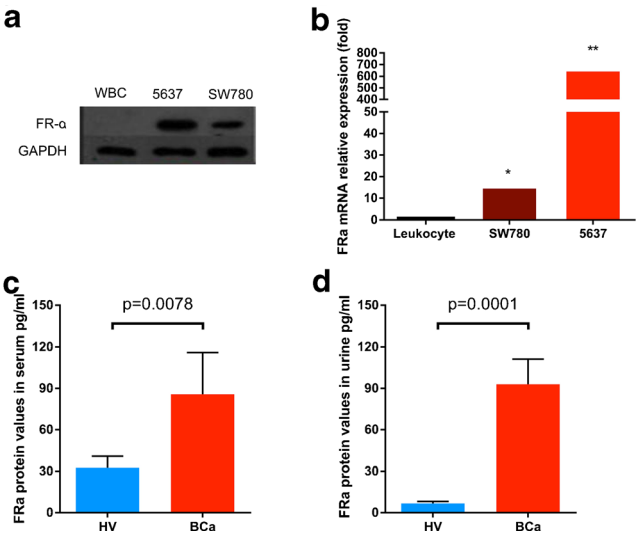

Fig. 1 Expression levels of folate receptor alpha in TCC cell lines and in the serum and urine of the bladder TCC patients and healthy volunteers. **a** Expression level of folate receptor alpha (FRα) protein in bladder TCC cells (5637 and SW780) was much higher than that in the leukocyte. **b** Comparison of expression level of FRα mRNA in bladder TCC cells and the leukocyte collected from the bladder TCC patients. The FRα mRNA value of bladder TCC cell lines was significantly upregulated (*$p<0.05$, **$p<0.01$). **c** FRα protein values in the serum. **d** FRα protein values in the urine

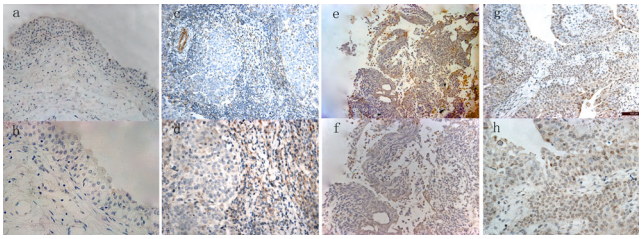

Fig. 2 Immunohistochemical analysis of the expression of FRα protein. FRα is mainly localized within the nuclei and cytoplasm. Immunostaining of the adjacent normal tissue samples (**a** ×200, **b** ×400) and the bladder TCC tissue samples: negative or weak FRα staining in cancerous tissue (**c** ×200, **d** ×400), moderate FRα staining in cancerous tissue (**e** ×200, **f** ×400), and strong FRα staining in most of tumor cells (**g** ×200, **h** ×400)

the bladder TCC cells but was little expressed in normal bladder tissue (Fig. 2).

PCR calibration and CTC unit

As an external calibration curve, six standards containing a serial of concentrations of the conjugated oligonucleotides were used to calculate the quantity of folate receptors on CTCs. To extrapolate the quantity of CTC from the quantity of folate receptors, an arbitrarily unit named CTC unit (Cu) were defined by the kit manufacturer GenoSaber Biotech Co., Ltd. (Shanghai, China). As determined by their calibration curve, the quantity of folate receptor number on 1×10^5 human nasopharyngeal cancer cell line KB cells is 7.5×10^{-14} mol, which is defined as 1×10^5 Cu. The detectable range of the assay is from 2 to 2×10^5 Cu. For quality controls, the intra-assay coefficient of variability (CV) is from 2.6 to 3.8 %, and inter-assay CV is from 3.3 to 5.3 %. Furthermore, based on manufacturer's demonstration, the average CTC recovery ratio was over 85 %, and the regression range was 0–10,000 tumor cell/mL with $R^2=0.9946$.

CTC values in patients with clinical and pathologic characteristics

Blood samples of 63 BCa patients were collected between October 2012 and September 2013. A total of six samples were excluded due to incomplete clinical information or poor quality of sample or other factors that may cause statistics bias. Thus, in total, the blood samples of 57 bladder TCC, 48 healthy volunteers, and 15 subjects with benign urologic pathologies were analyzed successfully. Table 1 shows the characteristics of these subjects and their CTC values. We observed that bladder TCC patients presented higher CTC values than healthy individuals ($p<0.001$). We observed no differences in CTC values considering gender, operation, recurrence, metastasis, and tumor grade.

ROC curve analysis to calculate the best cutoff point

Overall diagnosis performance of this method

We do the nonparametric ROC curve analysis to calculate the best cutoff point to discriminate between malignant and nonmalignant groups and further analyze the sensitivity and specificity of the assay for detection of bladder TCC. Area under the curve (AUC) for bladder TCC detection was 0.819. Figure 3a shows that when the cutoff threshold between the control group (benign patients and healthy volunteers) and cancer group was 18.19 Cu/3 mL, the test has the max Youden's index (0.556, $p<0.001$).

Operating characteristics of CTC units suitable for screening of bladder TCC patients

Sensitivity, specificity, and accuracy for bladder TCC detection were calculated for each Cu cut-point value. The resulting matrix of cut points and the corresponding percentages of sensitivity, specificity, and accuracy were plotted to represent the three observed parameters over the specific range of Cu cut-point interval. A screening tool should be characterized by high sensitivity, and thus, we marked 15.43 Cu/3 mL as the cutoff point, which yielded a higher sensitivity (82.14 %) (Fig. 3b).

In addition, the+LR, −LR, +PV, and −PV of this test for bladder TCC are 2.16 % (95 % confidence interval (CI) 1.7–

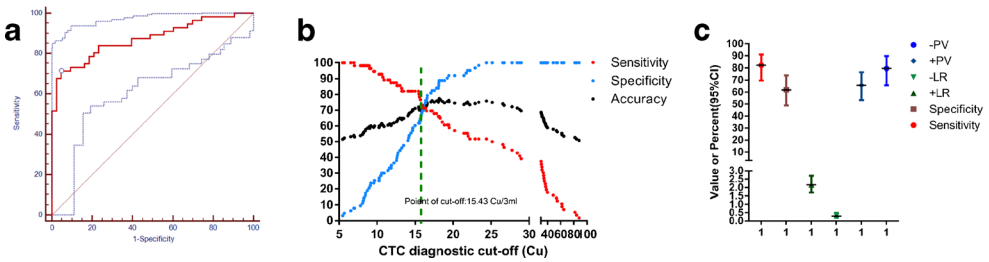

Fig. 3 Receiver operating characteristic (ROC) curve analysis to calculate the best cutoff point. **a** ROC analysis between the bladder TCC group and benign disease and healthy group. **b** Curves of relative diagnostic sensitivity, specificity, and accuracy for detection of bladder TCC according to different Cu cut-point values. **c** The diagnostic parameters of this method. The cutoff threshold was set as 15.43 Cu/3 mL

2.7), 0.29 % (95 % CI 0.2–0.5), 65.7 % (95 % CI 53.4–76.6), and 79.6 % (95 % CI 65.7–89.7), respectively (Fig. 3c).

Discussion

To the best of our knowledge, there is no research reporting the expression pattern of FRα in bladder TCC. Therefore, the application of FRα as the tumor marker to detect CTCs through tumor-specific ligand PCR is a fairly innovation for bladder TCC diagnosis.

As we discussed in the "Introduction" section, early diagnosis and initiation of appropriate therapy are essential for increasing the life expectancy of bladder TCC patients. However, the sensitivity of urinary markers for bladder TCC detection, including BTAtrak, NMP22, Immunocyt, and CYFRA 21.1, did not significantly exceed 60 % [17–19]. In addition, although cytology is frequently utilized, the even better performance is only shown in patients with high-grade lesions. It suffers from poor sensitivity for low-grade tumors [20–27]. While the gold standard for the diagnosis of BCa is based on the histopathologic evaluation of cystoscopy, an invasive procedure may be associated with significant cost and discomfort and, in some cases, may lead to important complications, such as infections and trauma [28, 29]. Consequently, these biomarkers and methods are not adequate for early screening [30]. The results of this study have shown that the FRα is a potential surface marker in identifying CTC of bladder TCC patients. The performance characteristics of our method, especially the specificity and sensitivity for low-grade tumor, are better than the most currently used method for the detection of bladder TCC. It is a superior early screening method for bladder TCC.

The overall sensitivity of current CTC detection assay was low, which was previously reported as 35.1 % (95 % CI, 32.4–38 %) [2]. In contrast to traditional methods, the sensitivity of our method to detect bladder TCC was much higher (82.14 %). Furthermore, it is almost noninvasive and can be combined with imaging examination for bladder TCC screening.

Concerning pathology stage and histopathologic grade, we observed no differences in CTC value (p=0.335 and p=0.575, respectively). Even though the recurrent patients (32.8 Cu, 95 % CI 16.1–49.0) had higher CTC value (Cu/3 mL) than the incipient patients (22.0 Cu, 95 % CI 18.1–29.0), no significant difference was found (p=0.134). These may be due to the lack of samples. For the same reason, there is a relatively low CTC value (median Cu is 16.5, 95 % CI 9.5–31.4) in the lymph or distant group (n=7).

To further confirm the results of our study and significance of this new method, the number of tested samples and the follow-up time in this research are still to be increased. Clearly, before this assay is used in changing the guidelines to manage bladder cancer, it is crucial to know how accurately FRα classifies or predicts outcomes [31, 32].

Application of tumor-specific ligand PCR in detecting circulating tumor cells have fine overall performance and is a promising noninvasive diagnostic method for bladder TCC. Because of its high sensitivity, it has great potentiality for bladder TCC screening.

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Conflicts of interest None.

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