Multiple Fluorescences in Situ Hybridization Status in Excreted Urine Improve Diagnostic Efficacy for Upper Urinary Tract Urothelial Carcinomas

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Purpose: To evaluate the diagnostic accuracy of single and multiple fluorescence in situ hybridization (FISH) tests for upper urinary tract cancer (UTUC), we analyzed the diagnostic efficacy of FISH in patients with UTUC and the difference between it and the Tumor Node Metastasis (TNM) stage and grade of the tumor.

Materials and Methods: Patients treated for UTUC at our institution between 2011 and 2021 who had not been previously diagnosed with UTUC were included. Patients were divided into single, two, and multiple (three times or four times) FISH groups based on the number of FISH tests performed on different samples from the same patient, and the diagnostic efficiency of single, two, and multiple FISH tests for muscle-invasive tumors and high-grade tumors were assessed.

Results: We included a total of 207 patients with UTUC, and when compared to single FISH, the sensitivity of multiple and double FISH for the diagnosis of UTUC increased from 62% to 76% and 78%, respectively. It went from 67% to 78% and 80% for muscle-invasive UTUC (> = pT2) and from 71% to 79% and 81% for the highest-grade UTUC.

Conclusion: Multiple FISH improves the diagnostic efficacy of UTUC and helps to differentiate aggressive tumors.

Keywords: fluorescence in situ hybridization (FISH); upper tract urothelial carcinoma (UTUC); high grade; radical nephroureterectomy (RNU); conservative management.

INTRODUCTION

Among uroepithelial carcinomas, upper urinary tract urothelial carcinomas (UTUCs) are a rare tumor type, accounting for only 5-10% of uroepithelial carcinomas[(1)]. Notably, more than 60% of UTUCs are invasive at diagnosis, while approximately 7% of UTUCs have distant metastases at the time of diagnosis[(2)]. Given the high recurrence rate and mortality of UTUC, radical nephroureterectomy (RNU) combined with cystic cuff resection has been the standard procedure for the treatment of high-risk UTUC[(3)]. For low-risk (small, low-grade, non-muscle-invasive) UTUC, adjuvant, and ablative intracavitary therapies, may be used to enhance renal function[(4)].

Platinum-containing neoadjuvant chemotherapy is known to be beneficial for patients with muscle-invasive bladder cancer but not for advanced or metastatic UTUC[(5)]. However, its clinical and prognostic effects in UTUC have not been elaborated. Recently, it has been shown that neoadjuvant chemotherapy with gemcitabine and cisplatin can improve the prognosis of T3 UTUC, but its long-term effects need to be further investigated[(6)].

Thus, early risk assessment of UTUC patients is essential to guiding the patient's later treatment options. However, determining the grade and risk level of UTUC from the patient's symptoms and imaging is very difficult.

Computed tomography urography (CTU) has the highest diagnostic accuracy of the imaging techniques available for the diagnosis of UTUC, with a summary specificity of up to 95%[(7)]. Magnetic resonance urography (MRU) is indicated for patients who are not candidates for CTU, in contrast to MRU, which has a lower sensitivity and specificity[(8)]. Flexible ureteroscopy (URS) allows for direct biopsy of lesions that can be biopsied; however, recent studies have demonstrated a risk factor for recurrence in the bladder when a biopsy is performed during URS[(7,9)].

Besides the laboratory, imaging, and endoscopic processes, attempts have been made to find new diagnostic methods to diagnose UTUC[(10)].

FISH is one of the diagnostic techniques. FISH is a method that uses fluorescently labeled DNA probes to detect cellular chromosomal variants[(11)].

FISH has become a routine test for the initial diagnosis and follow-up monitoring of UTUC due to its high

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Table 1. Clinical and pathological characteristics of UTUC patients according to single FISH status

□		Single FISH Positive	Negative	*P*-Value
Median age (years)		68.248 ± 10.684	70.269 ± 9.874	0.734
Patients		129 (32.32%)	78	
Gender	Female	79 (61.24%)	48 (61.54%)	0.966
	Male	50	30	
Hydronephrosis	Present	58 (44.96%)	43 (55.13%)	0.156
	Absent	71	35	
Tumor location	Pelvis	8 (6.20%)	7 (8.97%)	0.734
	Ureter	108(83.72%)	64 (82.06)	
	Both	13 (10.08%)	7 (8.97%)	
Tumor stage	A	3 (2.33%)	7 (8.97%)	0.046
	1	42 (32.56%)	30 (38.46%)	
	2	35 (27.13%)	24 (30.77%)	
	3	39 (30.23%)	15 (19.23%)	
	4	10 (7.75%)	2 (2.56%)	
Tumor grade	Low	11 (8.80%)	18 (37.69%)	0.001
	High	114	47	
Lymph node status	N +	16 (12.40%)	7 (8.97%)	0.447
	cN0 or pN0	113	71	
Distant transfer	M+	13 (10.08%)	5 (6.41%)	0.364
	cM0 or pM0	116	73	
Tumor size (cm)	<=3	88 (68.22%)	61(78.21%)	0.121
	>3	41	17	
Multifocal	No	110 (85.27%)	69 (88.46%)	0.408
	Yes	19	9	

[a]Data are presented as mean ± SD or number (percent)

sensitivity and specificity[12]. However, FISH results are influenced by many factors, and a negative FISH test alone does not guarantee the absence of significant disease, as a patient's tumor may be a "FISH invisible" lesion[13].

Here, we attempt to prove the significance of repeated independent FISH testing by comparing the variability of single as well as two or more independent FISH test results against different samples for UTUC, which can contribute to the diagnosis of the disease and predict tumor grading and TNM staging, giving the most appropriate treatment for patients.

MATERIALS AND METHODS

Inclusion and exclusion criteria

Between January 2012 and December 2021, a total of 230 patients with UTUC were admitted to the Department of Urology, Zhongnan Hospital of Wuhan University, and these patients were included in this study. 23 patients were not included in our group due to the absence of pathological findings or pathological confirmation of concurrent bladder cancer. So we reviewed the clinicopathological data of all the remaining 207 UTUC patients. Imaging and biopsy results for these patients confirmed their diagnosis. In 17 of these patients, tumor infiltration could not be clarified in pathological testing because tumor tissue was scarce or not taken at the time of biopsy, so we evaluated the TNM stage of their tumors based on their imaging data. For patients with high suspicion of UTUC, the interval between FISH tests should not exceed 7 days. Additionally, all tests are completed within one hospitalization cycle, with a total duration of no more than 4 weeks. In addition, our samples were collected on the same day and immediately sent for testing. All patients did not receive chemotherapy or radiotherapy before undergoing pathology or surgery. The Ethics Committee of Wuhan University Central South Hospital approved the study.

Treatment and evaluation

175 patients underwent RNU with ipsilateral bladder cuff excision. We do not routinely perform lymph node dissection intraoperatively, Only for patients in the localized progressive phase (T3/T4 or N+). After surgery, we perform routine bladder irrigation to reduce the incidence of postoperative bladder cancer. The remaining 32 patients did not undergo surgical treatment at our institution due to personal reasons; their tumor grading and staging are determined by a combination of imaging and biopsy findings. All pathological specimens were re-examined by a specialized genitourinary pathologist to confirm the initial diagnosis. Tumor staging was based on the 2009 TNM assessment of the International Union Against Cancer (UICC). Tumor grading was based on the 2004 World Health Organization classification and grading system. The tumor location was classified as the ureteral, renal pelvis, or both. Tumor multiplicity was defined as the simultaneous presence of two or more pathologically confirmed tumors in any location. We classified tumor size into two groups based on the median value of tumor size in patients using a cut-off of 3 cm. The status of regional lymph nodes was based on the final pathological specimen. For patients with advanced tumors, lymph node metastases, post-operative recurrence, or intolerance to surgery, we can perform a comprehensive evaluation and administer appropriate adjuvant therapy such as platinum-based chemotherapy, external radiation therapy, or immunotherapy.

Criteria for positive FISH

All samples were processed according to the UroVysion kit instructions. The time interval between different tests is not determined. All duplicate FISH assays were repeated with independent samples from the same patient at different times.

A brief description of the UroVysion kit is as follows: 20-50 mL of fresh urine is mixed with polyethylene glycol in a 2:1 ratio and stored in a 4°C refrigerator for

Table 2. Clinical and pathological characteristics of UTUC patients according to twice FISH status

□		twice FISH Positive	Negative	*P*-Value
Median age (years)		68.905 ± 10.729	69.347 ± 9.395	0.182
Patients		158 (76.33%)	49	
Gender	Female	93 (58.86%)	34 (69.39%)	0.186
	Male	65	15	
Hydronephrosis	Present	76 (48.10%)	25 (51.02%)	0.721
	Absent	82	24	
Tumor location	Pelvis	9 (5.70%)	6 (12.24%)	0.293
	Ureter	133 (84.18%)	39 (79.59%)	
	Both	16 (10.12%)	4 (8.16%)	
Tumor stage	A	5 (3.16%)	5 (10.20%)	0.037
	1	56 (35.44%)	16 (32.65%)	
	2	40 (25.32%)	19 (38.78%)	
	3	46 (29.11%)	8 (16.33%)	
	4	11 (6.96%)	1(2.04%)	
Tumor grade	Low	15 (10.49%)	14(29.79%)	0.001
	High	128	33	
Lymph node status	N +	18 (11.39%)	5 (10.20%)	0.817
	cN0 or pN0	140	44	
Distant transfer	M+	16(10.13%)	2 (4.08%)	0.19
	cM0 or pM0	142	47	
Tumor size (cm)	<=3	116 (73.42%)	33 (67.35%)	0.408
	>3	42	16	
Multifocal	No	136 (86.08%)	43 (87.76%)	0.764
	Yes	22	6	

same-day processing. The urine was centrifuged at 500 r/min for 10 min to obtain urinary exfoliated cells. The precipitate was fixed with a methanolic glacial acetic acid mixture and then centrifuged twice. The precipitated cells were resuspended with 15 μL of fixative to make cell smears. The cells were then identified with a fluorescent probe, hybridization was completed overnight at 37°C in a humid environment, rinsed, and air dried, followed by counterstaining with 4',6'-diamidino-2-phenylindole II, and the results were subsequently analyzed on fluorescence microscopy.

Result interpretation: Primarily GLP P16 site-specific probes and CSP3/CSP7/CSP17 chromosome attachment site-specific probes were detected. 100 cells per probe were counted simultaneously. In the CSP3/CSP7 locus-specific probes, CSP3 showed a red signal and CSP7 showed a green signal. If the number of abnormal cells specific to the CSP3 locus or CSP7 locus is >10%, then it will be considered FISH-positive. Similarly, GLP P16 and CSP 17 are shown as red signals and CSP 17 as green signals, and if the number of abnormal cells with CSP 17 >10% or GLP P16 >15% was considered FISH-positive, All FISH test kits are from Beijing GP Medical Technologies, Ltd.(#F01008-00). The details of the display chart about the FISH results are shown in **Figure 1**.

Statistical analysis

Independent-sample Mann-Whitney U and chi-squared tests were used to compare continuous and categorical variables, respectively. Binary logistic regression was used to evaluate preoperative factors. Sensitivity and

Table 3. Clinical and pathological characteristics of UTUC patients according to multiple FISH status

□		multiple FISH Positive	Negative	*P*-Value
Median age (years)		68.944 ± 10.685	69.239 ± 9.483	0.198
Patients		161(77.78%)	46	
Gender	Female	95(59.00%)	32 (69.57%)	0.195
	Male	66	14	
Hydronephrosis	Present	77(47.83%)	24 (52.17%)	0.603
	Absent	84	22	
Tumor location	Pelvis	10(6.21%)	5 (10.87%)	0.555
	Ureter	135(83.85%)	37 (80.43%)	
	Both	16(9.94%)	4 (8.70%)	
Tumor stage	A	5(3.11%)	5 (10.87%)	0.017
	1	56(34.78%)	16 (34.78%)	
	2	41(25.46%)	18 (39.13%)	
	3	48(29.81%)	6 (13.04%)	
	4	11(6.83%)	1(2.17%)	
Tumor grade	Low	15(10.27%)	14 (31.82%)	< 0.001
	High	131	30	
Lymph node status	N +	18	5 (10.87%)	0.953
	cN0 or pN0	143	41	
Distant transfer	M+	16(9.94%)	2 (4.34%)	0.235
	cM0 or pM0	145	44	
Tumor size (cm)	< =3	119(73.91%)	30 (65.22%)	0.247
	> 3	42	16	
Multifocal	No	139(86.34%)	40(89.96%0	0.913
	Yes	22	6	

Table 4. Sensitivity and positive predictive value of single, two, and multiple FISH positive status for predicting high-grade and muscle-invasive UTUC

□	□	single	twice	multiple
Muscle-invasive cancer (≥ pT2)	Sensitivity (%)	67.20 (58.97-75.43)	77.60 (70.3-84.9)	80.00 (72.99-87.01)
	Positive predictive value(%)	65.12 (56.9-73.34)	61.40 (53.81-68.99)	62.1(54.6-69.6)
High-grade cancer	Sensitivity (%)	70.81(63.79-77.83)	79.50 (73.26-85.74)	81.3 (75.3-87.3)
	Positive predictive value (%)	91.20 (86.23-86.17)	89.50 (84.48-94.52)	89.720 (84.82-94.62)

positive predictive values were calculated using the pooled results of FISH as a diagnostic test, and sensitivity and positive predictive values were calculated for tumor TNM staging or grading (high versus low). All statistical analyses were performed using SPSS version 26.0 (IBM Corp, Armonk, NY). All reported *P* values were bilateral. Statistical significance was set at 0.05.

RESULTS

All patients with UTUC underwent FISH, and since a positive result in just one test represented a positive final result, we divided the patients into 3 groups: single, twice, and multiple (three times or four times) according to the number of FISH tests. A total of 127 male and 80 female patients were enrolled in the study, and all patients had pathological results confirming UTUC. In 17 of these patients, the tumor grade could not be determined due to the limitations of the pathological examination (This item in the table only has 190 patients). For multiple FISH tests, the final result is one positive result. Comparing the results of single, twice, and multiple, we noted that the number of positive results increased to 158 for twice and 161 for multiple compared to 129 positive results for a single, and the sensitivity for UTUC diagnosis increased from 62% to 76% and 78%, respectively. Also, we conducted a univariate analysis, which showed that the difference between FISH test results and preoperative factors such as age, gender, and hydronephrosis is not significant. However, there was a significant difference between FISH test results and tumor grading and staging in these three groups. For single, twice, and multiple FISH tests, the *P*-values of tumor TNM stage were 0.046, 0.037, and 0.017; while the P-values for tumor grade were .001 (**Tables 1-3**). Compared with single FISH, the sensitivity of twice and multiple FISH for muscle-invasive UTUC increased from 67% to 78% and 80%, and the sensitivity of twice and multiple FISH for highly graded UTUC increased from 71% to 79% and 81%. This indicates that the diagnostic rate of UTUC increased significantly as the frequency of FISH testing increased. We further calculated the differences between FISH and other preoperative factors in the three groups by binary logistic regression analysis (Supplementary Table 1), and in the univariate analysis, there is no significant difference between the results of FISH and preoperative factors such as age, gender, and hydronephrosis. Similarly, we found no difference between FISH results and tumor TNM stage (low stage: <pT2, high stage: >=pT2) in binary logistic analysis, while there was still a significant association between FISH results and tumor grade. The *P* values for single, twice, and multiple are 0.005,0.005 and 0.004. In addition, we also conducted Cohen's kappa coefficient analysis on three sets of FISH, and the

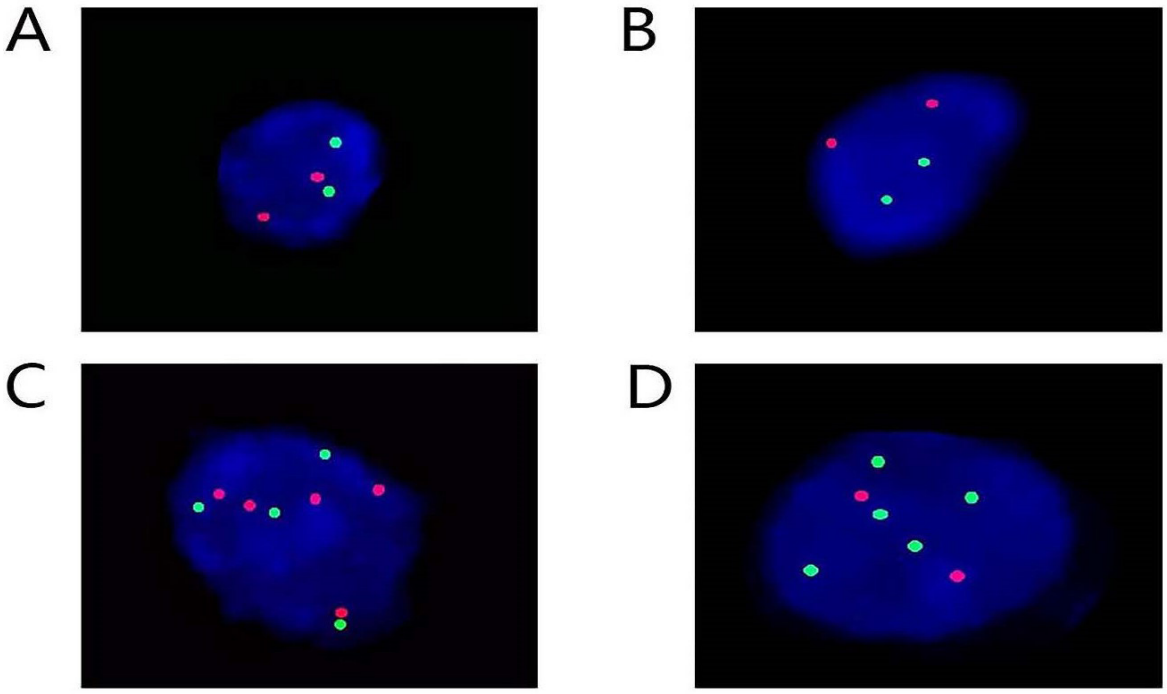

Figure 1. Representative FISH images. **(A)** a normal urothelial cell with diploidy of chromosomes 3 (red signal) and 7 (green signal), **(B)** a normal urothelial cell with diploidy of p16 genes (red signal), and chromosome 17 (green signal), **(C)** a cell with polyploidy of chromosomes 3 and 7, and **(D)** a cell with polyploidy of chromosome 17 and homozygous deletion of p16 genes

results showed that the coefficients of single VS twice, single VS multiple times, and two VS multiple times were 0.656, 0.619, and 0.959, respectively. This indicates that the diagnostic consistency of the three sets of FISH detection for UTUC is strong.

Among all UTUC patients, the FISH status was stratified by the pathological stage and grade of the patients (**Table 4**). For muscle-invasive UTUC, the sensitivity of the three groups of results was 67%, 78%, and 80%, while their positive predictive value (PPV) was 65%, 61%, and 62%. Respectively, the sensitivity for highly graded UTUC was 71%, 79%, and 81%, their PPV was 91%, 89%, and 90%, and the sensitivity values of single FISH and multiple FISH (combining secondary and tertiary results) were significantly different.

DISCUSSION

As early as 2014, the European Association of Urology (EAU) stated that RNU is no longer considered the gold standard treatment for all UTUC patients, and developed a low-risk model for UTUC[14].In addition, Su et al.[15] noted that treatment should preserve renal function in patients with UTUC with isolated kidneys and bilateral lesions with poor renal function. However, assessing the risk of UTUC preoperatively is a huge challenge, and an accurate preoperative assessment is directly related to the patient's benefit[16]. In conclusion, there is an urgent need for an accurate and easy-to-use detection method to predict the risk level of UTUC.

Urine cytology was used earlier to detect UTUC as a simple and easy-to-perform test. In 2004, Hofland et al. found that urine cytology has some diagnostic value for imaging and cystoscopy of missed cancers[17]. A further study by Mishriki et al. found that urine cytology's sensitivity for UTUC was 45.5% and its specificity was 89.5%, from which only 2 patients benefited[18]. Similarly, a recent multicenter study of 567 patients showed that the sensitivity of urine cytology for UTUC was still low at 43.5%[19]. The US Food and Drug Administration (FDA) has long-approved tests such as NMP22 (Nuclear matrix protein 22) and BTA (Bladder tumor antigen) as ancillary tests for bladder cancer[20]. Unlike the mechanism of chromosome detection by FISH, they rely on antigenic antibody reactions. Gong et al. investigated the diagnostic value of novel UTUC markers: BTA, Survivin, and NMP22 for UTUC. They found that the diagnostic efficacy of the three tests alone was low for UTUC, but the combination of the three tests could improve sensitivity and specificity[21]. These results show that urine cytology has good specificity but cannot make a definitive diagnosis of UTUC or determine its risk level. In addition, some studies have found that the methylation of urine DNA has a certain predictive effect on the prediction of early bladder cancer and recurrent bladder cancer, but at present, the relevant research is insufficient or not accurate enough to be used alone[22,23].

Current imaging methods used to detect UTUC include diffusions such as diffusion-weighted magnetic resonance urography, intravenous pyelography, and CTU, among which CTU has become the preferred diagnostic and staging modality[24]. A recent study by Florian included 1233 patients, and the analysis yielded a sensitivity and specificity of CTU for UTUC of 92% and 95%[25]. However, CTU may not accurately estimate disease invasiveness, which may lead to misclassification and thus compromise treatment[26].

Ureteroscopy can be used to visualize lesions and biopsy suspicious tissue. In a 2013 Claudia et al. study, a retrospective analysis of 54 patients showed that URS accurately predicted the grading of resected samples with a sensitivity of 92.6%, independent of sample size[27]. However, URS is handled as an invasive procedure with the usual risks of anesthesia, surgery, and complications such as ureteral avulsion, ureteral wall injury, bleeding, and perirenal hematoma[28]. In addition, studies are reporting an increased risk of intravesical tumor recurrence after undergoing URS[29,30].

According to a past study, the FISH assay is widely used in diagnosing various diseases, and in UTUC, the test depends on a large number of occurrences of specific chromosomal abnormalities[31]. A prospective study on the detection of UTUC by FISH was conducted by an institution back in 2016, and they found that the sensitivity of FISH for UTUC was significantly better than cytology[32]. In the same year, Gomella et al. studied the results of FISH in 415 patients, and the sensitivity of the FISH test increased from 42.4% to 54.9% compared to cytology[33]. It was also noted that FISH could be performed on a selective 1 ml urine specimen, and they found that FISH was feasible in all samples with a sensitivity and specificity of 90% and 80%, respectively, compared to 80% and 67% of the sensitivity and specificity of cytology[34]. In addition, the diagnostic performance of FISH testing was further explored by Eismann and Su et al.[12,35], Su found that the sensitivity and PPV of FISH were 76.5% and 59.5% for high-grade UTUC and 71.7% and 58.0% for muscle-infiltrating UTUC, respectively. These data suggest that FISH results can predict UTUC and may be useful for staging the diagnosis. A prospective study on the cost of FISH testing by Gayed et al. showed FISH testing avoids the costs incurred by biopsy and saves on treatment costs[36]. To obtain the most appropriate treatment for UTUC patients, the 2020 update EAU guidelines clearly state that it is useful to "risk stratify" UTUC between low-risk and high-risk tumors to identify those patients likely to benefit from kidney-sparing treatment[1]. Therefore, accurate diagnosis is very important for the treatment of UTUC patients.

In this study, we found differences in different independent repeat FISH tests in some patients and, more surprisingly, positive independent repeat FISH tests in some patients with negative first FISH tests and negative ureteral biopsies. Combining the data again, we found that independent repeat FISH improved the diagnostic efficiency of UTUC and had predictive value for TNM staging and tumor grading.

However, our study still has some limitations. First, this study was a single-center study with a small sample size and additionally, we had 17 patients whose tumor stage was defined by imaging. Second, selection bias may have influenced the results of this nonrandomized, retrospective study. Again, the interference of multiple preoperative factors resulted in the final treatment (conservative treatment or surgical treatment) not being based on preoperative FISH results. Moreover, we failed to combine the FISH assay with the markers indicated by the FDA, and the molecular mechanism of this needs to be further investigated. Second, the correlation between FISH and TNM staging of tumors in binary logistic regression analysis was not as close in the uni-

variate analysis due to the limitation of the data; it did not support the construction of AUC curves to develop further analysis. Data on the interval of FISH testing was lacking, and the issue of time interval needs to be further explored. Finally, the relationship between FISH results and tumor prognosis could not be verified due to the lack of follow-up data. Despite the many problems with the appeal, our results are sufficient to demonstrate that repeated independent FISH testing improves the diagnostic efficacy of UTUC and provides a reference for tumor TNM staging and grading. Therefore, combined with the higher price of FISH, we suggest that a second independent FISH test is necessary for patients with a high suspicion of UTUC who have a negative single test or a negative ureteral biopsy.

CONCLUSIONS

In contrast to single FISH, multiple FISH improved the diagnosis of UTUC and was predictive of TNM staging and grading of the tumor. Helps to differentiate aggressive tumors, thus allowing patients to obtain the most appropriate treatment.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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