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Utility of Fluorescence *In Situ* Hybridization (FISH) to Sub-Classify Low-Grade Urothelial Carcinoma for Prognostication

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

AB 1 Yi Chen*
DE 2 Bo Tao*
B 3 Ya Peng
D 4 Weijiao Yang
B 1 Chuang Wang
D 1 Xuebao Xiang
A 1 Tianyu Zhang
B 1 Li Gao
C 5 Jiaoyu Yi
A 4 Xiangfu Zhou
AG 1 Jiefu Huang

1 Department of Urology, The Affiliated Hospital of Guilin Medical University, Guilin, Guangxi, P.R. China
2 Department of Orthopaedics, The Affiliated Hospital of Guilin Medical University, Guilin, Guangxi, P.R. China
3 Center Laboratory of Basic Medical Science, Guangxi Medical University, Nanning, Guangxi, P.R. China
4 Department of Urology, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, P.R. China
5 Shanghai Jiao Tong University, Shanghai, P.R. China

* These authors contributed equally to this work

Corresponding Author: Jiefu Huang, e-mail: 601163132@qq.com

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Background: Fluorescence *in situ* hybridization (FISH) is used widely to detect cancer levels, but its value in urothelial carcinoma remains unclear. The aim of this study was to use FISH to examine the urine specimens of low-grade urothelial carcinoma (UC) patients to determine the possibility of sub-classifying the prognosis of UC.

Material/Methods: We diagnosed 107 patients with low-grade UC using a UroVysion kit to detect chromosomes 3, 7, 17, and P16 in the urine. An average 46.6-month follow-up completed in January 2016 combined with the clinical follow-up data were evaluated with Spearman's correlation analysis to analyze the aberration of chromosomes in relation to the prognostication. Univariate and multivariate analysis using the Mantel-Cox log-rank test for overall, cancer-specific, and disease-free survival were used to determine the prognostic significance of CSP7/CSP17 and CSP3/GLPp16.

Results: In the 107 samples, 84 showed positive reaction in the FISH test. Furthermore, CSP7/CSP17 was found to be significantly related with age, tumor size, T stage, and tumor numbers, but not in CSP3/GLPp16. In addition, Kaplan-Meier analysis and Cox proportional hazards regression revealed a significant negative correlation between CSP7/CSP17 and survival, while CSP3/GLPp16 showed no significantly differences.

Conclusions: CSP7/CSP17 positivity on FISH test appears to play a critical role in low-grade UC and may be considered as a high-risk and prognosis factor.

MeSH Keywords: Carcinoma *In Situ* • Flucytosine • Prognosis

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Background

Urothelial carcinoma (UC) is one of the most common tumor diseases of the urinary system [1]. More than 70% of cases consist of non-muscle invasive bladder cancers (NMIBCs), including 70% of Ta and T1 carcinoma *in situ*. The risk assessment table of the European Organization for Research of Cancer (EORTC) of 2006 is considered the best assessment tool for NMIBC and is based on 6 clinical and pathological factors: tumor number, tumor size, prior recurrence rate, T stage, carcinoma *in situ*, and tumor grade [2]. According to the assessment, tumors are divided into 3 groups: a high-risk group (pT1, G3, multi-focal, and CIS), a low-risk group (pTa, G1, <3 cm diameter, focal), and other defined medium groups [3].

According to the EORTC, the consistency of pathologists in judging whether the tumor infiltrating through the mucosa lamina propria is low, especially in the G1, G2, and G3 judgements; this low level of consistency has a direct impact on patients undergoing surgery and those receiving postoperative drug perfusion treatment during follow-up [4]. Tumor pathological grade is the only assessment factor that considers tumor biological characteristics and also possesses great significance in the prognosis of urothelial carcinoma. However, both WHO1973 G2 and WHO 2004 low-grade UC cannot fully reflect the classification of tumor recurrence and progression.

Currently, cystoscopy and urinary cytology examinations are the most common methods for the detection of UC and to predict its recurrence and progression. Cystoscopy is referred to as "the gold standard" and is the most simple, economical, and efficient method of examination for monitoring postoperative NMIBCs. Cystoscopy, however, is an invasive examination; it may cause pain in some patients and has false-negative rates of 10–30% [5]. Although the urine cytology test has high specificity, the sensitivity of cytology is very low, especially for low-grade UC [6,7].

In recent years, fluorescence *in situ* hybridization (FISH) analyses, used to detect UC at the chromosomal level, including the aneuploidy of chromosomes 3, 7, 17, and 9 p21, has become a topic of strong interest [8], and several studies [9,10] have confirmed that the instability of genome analysis can be used as an independent factor to improve UC classification accuracy and to facilitate the evaluation of recurrence and progression of tumors. Chromosomes 7 and/or 17 positive are considered to be a high-risk group, and chromosomes 3 and/or p16 positive are a low-risk group generated in intermediate-risk urothelial carcinoma [11]. In our previous study, we discussed the relationship between FISH and prognosis of low-grade UC; we proposed using the FISH results to predict the risk of recurrence and progression by sub-classifying low-grade patients into low-risk and high-risk groups.

In the present study, we further explore the long-term follow-up of low-grade UC patients, assess the risk factors associated with FISH results, and discuss the possibility of using FISH technology to sub-classify and predict the prognosis of low-grade UC patients.

Material and Methods

Clinical data

We included 107 low-grade UC patients (age range=39–85 years, average age=65.1 years, median age=64 years, SD=11.3 years) from the Third Affiliated Hospital of Sun Yat-sen University and the Guilin Medical College Affiliated Hospital between 2009 and 2015. The patients were undergoing TURBT (Transurethral resection of bladder tumor) and received 30 mg pirarubicin within 6 h after resection. The postoperative follow-up was continued until January 2016, and the mean observation period was 46.6 months (range=6–80 months, median=47 months, SD 18.9 months). Postoperative follow-up evaluations were conducted every 3 to 6 months and included cystoscopy, and recurrence was defined as occurrence a new lesion of the same grade and stage. If the grade or stage was increased, the lesion was considered progressive. The tumor staging was evaluated based on the UICC standard [12]. All of the patients signed an informed consent form that had been approved by the Ethics Committee.

Urine collection and preparation and pretreatment of sample slides

Morning urine was collected continuously for 3 days before TURBT, centrifuged 10 min at 1500 rpm, and the supernatant was removed. The cell pellets were resuspended in collagenase solution B, followed by incubation in a hypotonic solution of potassium chloride (KCl) (0.075 M), and subsequently fixed with 2 ml of fixatives. Slides were prepared from the voided urine samples and aged. Subsequently, the slides were dehydrated in a series of ethanol washes (70%, 85%, and 100%, 2 min each), heated to 56°C and prepared for FISH.

FISH detection

FISH was performed according to the manufacturer's protocols (Beijing GP Medical Technologies, Ltd., Beijing, China). CSP3, CSP7, CSP17, and GLPp16 DNA probe cover Human chromosome 3, 7, 17, and p16 gene in chromosome 9, respectively. CSP3 probe and GLPp16 DNA probe is a red signal, whereas CSP7 probe and CSP17 probe are green. For slide preparation and denaturation, the prepared slides were immersed in denaturing solution at 76 for 5 min, then sequentially placed in pre-cooled 70% ethanol, 85% ethanol, and 100% ethanol for

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Table 1. The results of FISH test follow-up in 107 low-grade urothelial carcinoma cases.

	Recurrence		Progression		Total
CSP7/17 & CSP3/P16 (+)	10	(50.0%)	10	(50.0%)	20
Only CSP7/CSP17 (+)	13	(36.1%)	10	(27.8%)	36
Only CSP3/GLPp16 (+)	8	(28.6%)	4	(14.3%)	28
FISH negative	5	(21.7%)	1	(4.3%)	23

Table 2. Clinical data and the relationship between FISH results and risk factors of low-grade UC.

	Number (%)	CSP7/CSP17		P	CSP3/GLPp16		P
		+	–		+	–	
Age (years)							
≤65	56 (52.3)	23	33	0.014	22	26	0.224
>65	51 (47.7)	33	18		34	25	
Gender							
Male	65 (60.8)	35	30	0.697	33	32	0.126
Female	42 (39.2)	21	21		15	27	
Tumor size							
≥3 cm	57 (53.3)	35	22	0.045	29	28	0.181
<3 cm	50 (46.7)	21	29		19	31	
TNM grade							
Ta	79 (73.4)	35	44	0.005	32	47	0.128
T1	28 (26.6)	21	7		16	12	
Tumor number							
Focal	61 (57.0)	25	36	0.007	26	35	0.592
Multi focal	46 (43.0)	31	15		22	24	

3 min each to dehydrate. For probe mix preparation and denaturation, we used 10 µl of degenerated probe mixture (2 µl probe mix, 7 µl hybridization solution, and 1 µl water) added into a microcentrifuge tube. After centrifugation for 1~3 s, vortexed, and mixed again, then briefly centrifuged. The tubes containing more than 10 µl of the FISH probe mix were placed in a 76°C water bath for 5 min, then placed in a 46°C water bath and removed before hybridization. We added 10 µl of the probe mixture to the slide hybridization area and immediately covered it with a coverslip. We covered the edge of the glass slide with rubber sealant to avoid air bubbles between the coverslip and the glass slide. The slide was placed in a preheated wet box and hybridized overnight in a 42°C incubator. The post-hybridization washing was performed with a 50% formamide/2×SSC solution for 10 min, a 2×SSC solution for 10 min, a 0.1% NP-40/2×SSC solution for 5 min, and then 70% ethanol at room temperature for 3 min. We added 15 µl 4' and 6-diamidino-2-phenylindole dihydrochloride (DAPI) to the slide hybridization area and immediately covered it with a coverslip. After 15 min in the dark, visualization of the signals in the slides was performed with a computerized imaging system and a fluorescence microscope. Counting results were completed by 2 independent pathologists, and the results had to be agreed upon before they were considered valid.

Statistical evaluation

The clinical data and CSP7/CSP17 and CSP3/GLPp16 expression levels in 107 cases of low-grade urothelial carcinoma were analyzed by Spearman's correlation analysis. We also performed univariate Mantel-Cox log-rank test for disease-free, cancer-specific survival, and overall survival. Survival plots were drawn using Kaplan-Meier analysis to assess the significance of prognosis and FISH results. Stepwise multivariate Cox regression

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analyses was performed to assess disease-free, cancer-specific, and overall survival to determine the prognostic significance of FISH in chromosomes. Hazard ratios (HR) are shown with 95% confidence interval (CI), and P values <0.05 were considered statistically significant. All data were analyzed with IBM SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

Results

The results of FISH and patient follow-up

Among the 107 urine samples from low-grade UC patients, 84 were positive according to the FISH test, including 20 CSP7/CS17-positive and CSP3/GLp16-positive cases (18.7%); 36 cases (33.6%) were only CSP3/GLPp16-positive, 28 cases (26.2%) were only CSP7/CSP17-positive, and samples in 18 cases (21.5%) were negative (Table1). The 107 patients with low-grade UC after TURBT underwent postoperative follow-up evaluations using cystoscopy. Thirty-six patients had recurrence: 10 patients who were both CSP7/CSP17-positive and CSP3/GLPp16-positive, 8 were only CSP3/GLPp16-positive, 13 were only CSP7/CSP17-positive, and 5 were FISH-negative. Twenty-three cases had progression: 8 were both CSP7/CSP17-positive and CSP3/GLPp16-positive, 4 were only CSP3/GLPp16-positive, 10 were only CSP7/CSP17-positive, and 1 was FISH-negative).

The relationship between FISH results and risk factors of low-grade UC

As shown in Table 2, according to Spearman's correlation analysis, the expression of CSP7/CSP17 was correlated with age, tumor size, T stage, and tumor number (P=0.014, 0.045, 0.005, and 0.007, respectively), but not with sex (P=0.697). Nevertheless, CSP3/GLPp16 positivity was not associated with any risk factors in low-grade UC (P>0.05). These results suggest that CSP7/CSP17 plays a critical role in low-grade UC and may be considered as a high-risk factor.

The relationship between FISH results and prognosis of low-grade UC

Univariate Mantel-Cox log-rank test was used to analyze the overall survival of CSP7/CSP17-positive and CSP7/CSP17-negative cases. Statistically significant (chi-square=4.181, P=0.041, Figure 1A) differences were observed in the CSP7/CSP17 group. When CSP3/GLPp16-positive cases were compared with CSP3/GLPp16-negative cases, there was no significant difference (chi-square=0.043, P=0.836, Figure 1B). When CSP7/CSP17-positive and CSP7/CSP17-negative cases were compared for disease-free survival, a significant difference (chi-square=16.971, P<0.001, Figure 1A) was found, but not in the CSP3/GLPp16-positive and CSP3/GLPp16-negative groups (chi-square=0.911, P=0.340, Figure 1B). When compared in terms of cancer-specific survival, it was significantly different in the CSP7/CSP17-positive and CSP7/CSP17-negative cases groups (chi-square=5.903, P=0.015, Figure 1A) but not between the CSP3/GLPp16-positive and CSP3/GLPp16-negative cases groups (chi-square=0.562, P=0.454, Figure 1B).

In multivariate Cox regression analyses, we showed that CSP7/CSP17 positivity on FISH test in low-grade UC significantly affected overall survival (HR=2.306, 95%CI: 1.009–5.268, P=0.048), disease-free survival (HR=2.890, 95%CI: 1.654–5.051, P<0.001), and cancer-specific survival (HR=3.210, 95%CI: 1.184–8.703, P=0.022). No significantly result was found in CSP3/GLPp16-positive and CSP3/GLPp16-negative groups (P>0.05) (Table 3).

Discussion

In UC, histopathological grade is an important prognostic indicator for postoperative NMIBCs. It plays a key role in determining high, medium, and low risk of NMIBCs; however, neither the classification according to the 1973 WHO classification system nor the WHO 2004 histopathological classification system provide prognostic significance for tumor recurrence and progression. In addition, tumor pathological grading is often underestimated due to changes in tissue morphology that occur after the first transurethral resection of bladder tumors, the experience of surgeons, and subjective factors that influence the decisions of pathologists [13]. The postoperative prognosis of NMIBC has always been a difficult problem for urological surgeons.

The analysis of genomic instability can be used as an independent factor to improve the accuracy of UC classification and to facilitate the evaluation of recurrence and progression of a tumor.

Our research using FISH technology to detect low-grade UC shows that CSP7/CSP17 plays a crucial role and may be considered as a specific biomarker of poor prognosis. When CSP7/CSP17-positive cases are contrasted with CSP7/CSP17-negative cases, the former are more likely to exhibit recurrence and progression, leading to high mortality. We can infer that CSP7/CSP17-positivity indicates a high risk of recurrence and progression, whereas CSP3/16-positivity represents a low risk. Simonetti et al. [14] detected G1, G2, and G3 grade tumors in UC patients by FISH and found that chromosome 17 polyploidy was increased and was associated with the UC stage. The present study did not find any association with levels of risk of recurrence and progression and polyploidy because the number of included patients was small. Houskova et al. [15] examined the relationship between FISH and tumor grade in 128

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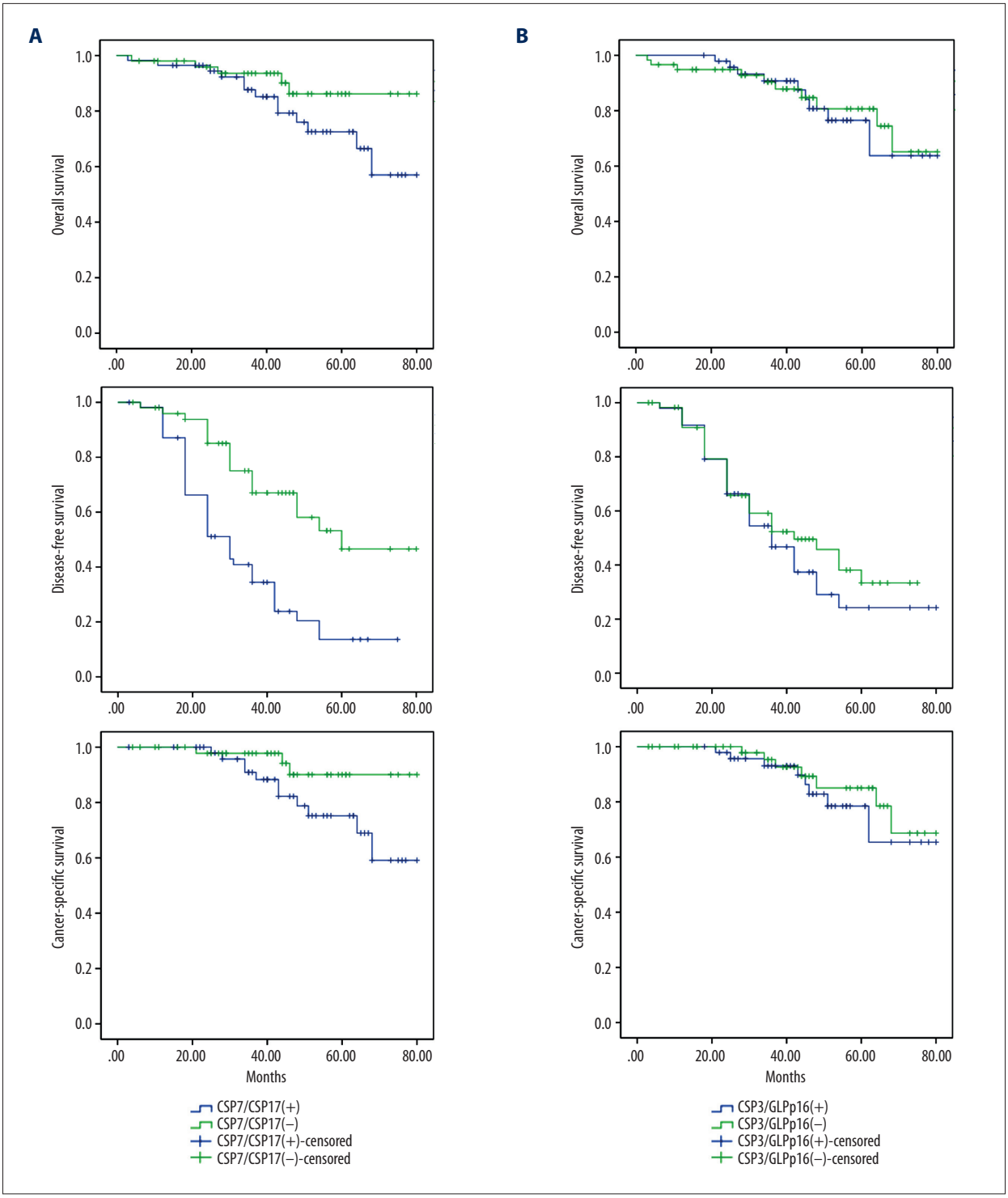

Figure 1. (**A**) Univariate Mantel-Cox log-rank test for overall survival, disease-free survival, and cancer-specific survival when comparing CSP7/CSP17-positive with CSP7/CSP17-negative patients (chi-square=4.181, P=0.041, chi-square=16.971, P<0.001, and chi-square=5.903, P=0.015, respectively). (**B**) Univariate Mantel-Cox log-rank test for overall survival, disease-free survival, and cancer-specific survival when comparing CSP3/GLPp16-positive with CSP3/GLPp16-negative patients (chi-square=0.043, P=0.836, chi-square=0.911, P=0.340, and chi-square=0.562, P=0.454, respectively).

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Table 3. Univariate and Multivariate analysis of survival.

Parameter	Univariate P	Multivariate analysis			
		P	HR	95% CI	
				Low	High
Overall survival					
CSP7/CSP17	0.041	0.048	2.306	1.009	5.268
CSP3/GLPp16	0.836	0.837	1.087	0.492	2.399
Cancer-specific survival					
CSP7/CSP17	0.015	0.022	3.210	1.184	8.703
CSP3/GLPp16	0.454	0.459	1.393	0.580	3.351
Disease-free survival					
CSP7/CSP17	0.00	0.00	2.890	1.654	5.051
CSP3/GLPp16	0.340	0.370	1.224	0.787	1.905

specimens and found that the G1, G2, and G3 positive rates were 63.6%, 64.3%, and 91.7%, respectively, suggesting that FISH may serve as a potential risk classification method for UC and that chromosome detection is an independent postoperative prognostic factor for UC patients. However, in our study, we also found 13 cases of postoperative recurrence and 5 cases of postoperative progression among the 51 patients in the CSP7/CSP17-negative group. This finding may be due to tumor analyses using other channels or the appearance of false-negatives in the FISH assay.

Our study found that when the CSP3/GLPp16 positive and CSP3/GLPp16 negative patients were compared, no significant differences were observed in prognosis. Matsuyama et al. [16] also used FISH to examine 118 UC patients and found that the absence of 9p21 genes was related to UC recurrence. The results of our study showed that when CSP3/GLPp16-positive patients were compared to CSP3/GLPp16-negative patients in risk groups, no differences in recurrence or progression were observed ($p>0.05$), which may be related to the low FISH detection sensitivity for low-grade UC (60-70%) [17]. This result may also have occurred because the CSP3/GLPp16 patients rarely exhibit malignant tumor biology and have less infiltration, which is more easily completely surgically resected. In addition, the finding may be due to the small number of cases.

In summary, the present study used FISH to detect low-grade UC in urine specimens of patients, and by combining this information with clinical data, we compared CSP7/CSP17 and CSP3/GLPp16 groups regarding the risk factors and prognosis of low-grade UC. We found that CSP7/CSP17-positivity defined a high-risk group, whereas CSP3/GLPp16 positivity shows no significance. This study confirmed that FISH detection of mutations in chromosomes 3, 7, 17, and 9p21 in urine samples can facilitate risk stratification of low-grade UC patients. CEP7/17-positivity was associated with recurrence and progression and poor survival, However, CEP7/17-negative postoperative patients were not likely to exhibit progression and recurrence. In addition, CSP3/GLPp16-positive patients should regularly undergo postoperative bladder microscopic screening to detect new tumors in a timely manner. This study provides a better theoretical basis for determining postoperative recurrence, which can facilitate individualization, risk assessment, and treatment.

Due to the small number of cases, the present study failed to show a relationship between UC prognosis and an increase in polyploidy. Our future studies will include more cases to explore the relationship between polyploidy and tumor malignancy to better understand risk stratification.

Conclusions

Our studies have showed that CSP7/CSP17 positive in FISH test appear to play a critical role in low-grade UC and may be considered as a high-risk and prognosis factor.

Conflict of interest

None

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