The Utility of Fluorescence in Situ Hybridization for Diagnosis and Surveillance of Bladder Urothelial Carcinoma

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Purpose: To evaluate the clinical value of fluorescence in situ hybridization (FISH) for diagnosis and surveillance of bladder urothelial carcinoma (BUC).

Materials and Methods: Between November 2010 and December 2013, patients suspected of having BUC were examined using urine cytology and FISH assay. Based on histopathological examination results, FISH results were compared with urine cytology. In addition, patients with a history of non-muscle invasive BUC were also examined using urine cytology and FISH assay at the first time of visit and then monitored with cystoscopy during follow-up period.

Results: A total of 162 patients included in this study and 12 patients were excluded due to uninformative FISH assays. The remaining 150 patients consisted of 108 patients suspected for BUC and 42 patients with a history of non-muscle invasive BUC. The sensitivities of FISH analysis and urine cytology were 72.8% and 27.2%, respectively, and the difference was statistically significant (P <.05). Difference between specificity of urine cytology (100%) and FISH assay (85%) was not statistically significant (P >.05). At the first visit, of 42 patients, one patient had positive cystoscopy, and FISH assay was positive in 26 of 41 patients with negative cystoscopy. During the follow-up period (mean, 29.5 months), 18 of 26 patients developed recurrence, and recurrence occurred in only one of 15 patients with negative FISH analysis.

Conclusion: Our results suggest that FISH analysis can be used as a non-invasive diagnostic tool for patients suspected of having new BUC. In addition, FISH analysis may provide important prognostic information to better define the individual risk for BUC recurrence.

Keywords: in situ hybridization; fluorescence; neoplasm recurrence; tumor markers; urinary bladder neoplasms; predictive value of tests; urine; cytology.

INTRODUCTION

Bladder urothelial carcinoma (BUC) is the fifth most common cancer in the world.[1] Approximately 80% of BUC is non-muscle invasive and 20% is muscle invasive. Most BUC tumors can be treated with transurethral resection of bladder tumor (TURBT) and additional intravesical chemo- or immunotherapy. However, patients with non-muscle invasive BUC have a significant risk of recurrence and progression to muscle invasive one.[2] Therefore, it is necessary to monitor recurrence and progression in patients with BUC using some modalities.

Cystoscopy and urine cytology have been the standard diagnostic and surveillance tools for BUC. However, cystoscopy is invasive and generally cannot be accepted by every patient. Meanwhile, some flat or very small lesions, such as carcinoma in situ, cannot be detected by cystoscopy.[3] Although urine cytology has high specificity and is a noninvasive method for detecting carcinoma cells, the procedure has poor sensitivity to tumor cells, particularly in low grade tumors.[4] Therefore, researchers have been looking for a noninvasive method with both good sensitivity and high specificity for detection and monitoring of BUC.

Previous studies have found a number of frequent genetic aberrations, such as aneuploidy of chromosomes 1, 3, 7, 9, 11 and 17 and deletions or the total loss of chromosome 9 in urothelial carcinoma (UC).[5,6] In addition, some studies have reported that some genetic mutations are usually associated with occurrence and development of BUC, such as Ki-67, Bcl-2 and CA199.[7,8] FISH technology is one of the standard methods for detecting these genetic mutations which uses fluorescently labeled DNA probes to detect numerical or structural abnormalities of the chromosomes in tumor cell. It has been demonstrated that multicolor FISH assay consisted of 4 probes for chromosomes 3, 7, 17 and P16 locus of chromosome 9 has

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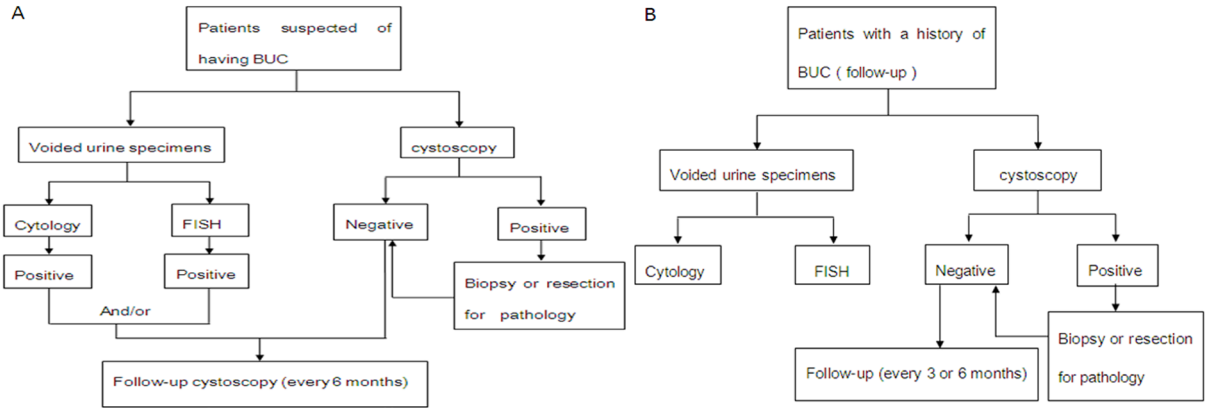

Figure 1. Study flow chart.

highest sensitivity for diagnosis of BUC from a series of 10 different probes.[9] Previous studies have reported that multicolor FISH assay as an ancillary tool could be used for diagnosis and surveillance of BUC.[10-14] However, some studies have reported that the sensitivity and specificity of FISH analysis to detect carcinoma were low,[15,16] and the value of FISH assay as a diagnostic tool and prognostic marker for BUC remains a controversial topic. In the present study, we aimed to compare the accuracy of FISH assay with urine cytology for diagnosing BUC and to determine the clinical implication of this assay for predicting the risk of BUC recurrence.

MATERIALS AND METHODS

Study Subjects

Between November 2010 and December 2013, patients suspected of having BUC, who presented with gross hematuria and an ultrasound diagnosis of suspicious bladder lesion before participating in the study were examined using both urine cytology and FISH analysis before doing cystoscopy. Patients with negative cystoscopy and positive urine cytology or positive FISH analysis underwent follow-up cystoscopy every 6 months (**Figure 1A**). Likewise, patients with a history of non-muscle invasive BUC were examined by urine cytology and FISH assay before cystoscopy at time of their first visits and then monitored with cystoscopy approximately every 3 months for 2 years with decreasing frequency thereafter to detect signs of recurrence of BUC (**Figure 1B**). The study was approved by our Institutional Review Board and all patients provided written informed consent.

Cytology Analysis and Pathological Examination

Urine cytology analysis was conducted by centrifuging a part of urine samples from whole volume of voided urine, and then using the Papanicolaou staining method. The used cytologic criteria were according to Modern Cytopathology.[17] Cytology results were interpreted by a trained cytopathologist who was blinded to the patients' clinical records and FISH results. Cytology results were considered positive only in cases where carcinoma cells or suspicious carcinoma cells were detected, while results were considered negative showing atypical or negative cells in three consecutive voided urines.

Histopathological examination was performed in patients with BUC who underwent TURBT or biopsy. Tumor stage and grade were determined according to the International Union Against Cancer TNM classification and the World Health Organization (WHO) 2004 classification method.

FISH Analysis

FISH assay was conducted using Bladder Cancer Kits (GP Medical Technologies, Ltd, Beijing, China) in accordance with the manufacturer's instructions as described elsewhere.[18] Briefly, the remaining urine specimen after cytological processing was collected from each patient and then centrifuged. Cells were mixed using fresh Carnoy fixative protocol, and the cell suspension was placed onto two slides and air dried. The slides were then washed in the 2 × standard saline citrate (SSC) and incubated in pepsin solution. They were then washed, fixed in methanol and dehydrated in 70%, 80% and 100%

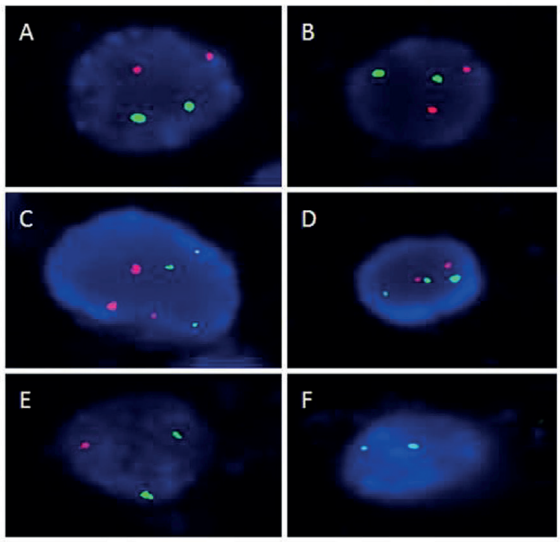

Figure 2. Fluorescence in situ hybridization; **(A and B)** normal cell showing two chromosomes 3 (green), two chromosomes 7 (red), two chromosomes 17 (green) and two 9p21 locus (red); **(C and D)** two examples ofabnormal cells showing aneuploidy of chromosome 3, 7 and 17; **(E and F)** two examples of abnormal cells showing heterozygous and homozygous deletions of the 9p21 locus.

ethanol, and then air dried. After DNA denaturation, two probe sets (CSP 3/CSP 7 and GLP p16/CSP 17) comprising chromosome 3 (Spectrum Green)/7 (Spectrum Red) and chromosome 17 (Spectrum Green)/specific locus p16 of chromosome 9 (Spectrum Red) were incubated with the specimens on the two slides and the cells were counterstained with DAPI (4, 6-diamidine, 2-phenylindole). Finally, specimens were analyzed using a fluorescence microscope equipped with the appropriate excitation and emission filters.

We have evaluated FISH results based on the criteria supplied by the Bladder Cancer Kit, using the following protocol. At least 100 consecutive cells were scored. An indicator was considered positive if the ratio of cells showing deletion of the p16 locus was ≧ 15% or the multiplication of CEP 3, CEP 7 or CEP 17 was ≧ 10%. A sample was considered FISH-positive if at least one of the following criteria was met: 1) at least 2 indicators were positive; 2) more than 15% of cells showed complete deletion of the p16 locus.

We have determined FISH results according to scanning method if the indicator fell below the percentages (10% or 15%) required to be considered indicator-positive, but was above certain thresholds (the mean percentage + 3 standard error [SD] of cells with polysomy or deletion observed in specimens from 20 normal donors). The thresholds for incomplete and complete loss of the p16 locus were 2.5% and 4.4%, respectively; for CEP 3, CEP 7 and CEP 17 the thresholds were 6.5%, 3.8% and 3.0%, respectively. Based on the scanning method, 25 morphologically abnormal cells with unclear enlargement, irregular nuclear borders and patchy DAPI staining were scored from each sample. Sample was considered FISH-positive if there were ≧ 4 cells with polysomy on at least 2 chromosomes (CEP 3, 7 or 17) or 12 cells showing homozygous loss of the P16 locus.

Statistical Analysis

The sensitivities of FISH assay and urine cytology were determined for patients with biopsy-proven BUC, and the specificities were calculated for patients diagnosed with benign disease. The significant differences between FISH assay and assay urine cytology results were determined using the McNemar test. Differences were considered statistically significant at P values < .05. The Statistical Package for the Social Science (SPSS Inc, Chicago, Illinois, USA) version 13.0 was used for statistical analysis.

RESULTS

A total of 162 patients were recruited in this study and 12 patients were excluded due to uninformative FISH assays. The remaining 150 patients consisted of 108 patients suspected of having BUC and 42 patients with a history of non-muscle invasive BUC. Of 108 patients suspected of having BUC, 81 had pathology confirmed BUC, 4 had ureter UC (located at the lower ureter, close to ureteral orifice) without bladder tumors and 3 had bladder adenocarcinoma. The remaining 20 patients were diagnosed with benign bladder disease, and served as the control group.

Among the 81 patients, 59 cases were FISH-positive (72.8%) (**Figure 2**), while only 22 cases had positive urine cytology (27.2%). The sensitivity of FISH assay was superior to that of urine cytology (P < .05). Of 20 control cases, FISH testing was negative in 17 and positive in 3 (2 glandular cystitis and 1 granulomatous cystitis), while all 20 cases had negative urine cytology, resulting in specificities of 85% and 100%, respectively. Although the specificity of urine cytology was higher than that of FISH assay, the difference was not statistically significant (P > .05). In addition, the positive predictive values (PPV) of FISH assay and urine cytology were 95.2% and 100%, respectively, while the negative predictive values (NPV) were 43.6% and 25.3%, respectively (**Table**). The sensitivities of FISH assay and urine cytology, broken down according to BUC stage and grade (**Table**). Among four patients with pathtologically confirmed ureter UC, three patients were FISH-positive, while only one had positive urine cytology result. All three patients with bladder adenocarcinoma were FISH-positive. Seventy-nine (97.5%) of the BUC cases were detected by cystoscopy and two cases were not detected, thereby to the sensitivity of cystoscopy was superior than that of FISH assay (72.8%) (P < .05). Among 42 patients with a history of BUC, follow-up cystoscopy detected a recurrent tumor in one patient; the tumor was pathologically confirmed as recurrent low-grade and non-muscle invasive BUC. Of 41 patients with

Table. Sensitivities, specificities and the predictive values of FISH assay and urine cytology for diagnosis of bladder carcinoma.

Variables	Patients No.	FISH Assay*	Urine Cytology*	*P* Value
Sensitivity				
Stage				
Non-muscle invasive	53	35 (66.0)	6 (11.3)	.000
Muscle invasive	28	24 (85.7)	10 (57.1)	.018
Grade				
Low	48	33 (68.8)	4 (8.3)	.000
High	33	26 (78.8)	18 (54.5)	.037
Total	81	59 (72.8)	22 (27.2)	.000
Specificity				
Controls	20	17 (85.0)	20 (100)	.72
PPV	.....	59/62 (95.2)	22/22	.29
NPV28	.....	17/39 (43.6)	20/79 (25.3)	.44

Abbreviations: FISH, fluorescence in situ hybridization; PPV, positive predictive value; NPV, negative predictive value.
* Data are presented as no. (%).

negative cystoscopy and negative urine cytology results at the time of the first visit, 26 patients were FISH-positive, and 18 of 26 patients developed recurrence during the follow-up period (mean, 29.5 months). There was recurrence in only one of 15 patients who was FISH-negative. Among the three patients with negative urine cytology and positive FISH assay in the control group, none of them was diagnosed with BUC or upper tract UC during follow-up period (mean, 28.2 months).

DISCUSSION

In the current study, we compared the accuracy of FISH assay with that of urine cytology to determine the clinical utility of this assay for detecting BUC. Our results indicated that the sensitivity value of FISH assay was significantly higher than that of urine cytology, regardless of the grade and stage of the disease, particularly in cases of low grade and non-muscle invasive tumors and there was no significant difference between the specificity of FISH assay and urine cytology, which are consistent with previous reports.[10,11,13,14]

Among the 59 patients who had negative cytology, 39 (66.1%) were FISH-positive. The FISH-positive result seems to provide a significant additional and complementary clue for detecting BUC when urine cytology results are negative or equivocal.[14] In 59 patients with negative urine cytology, 37 of 57 patients who had positive cystoscopy were also positive for FISH analysis, and the remaining 2 cases with negative cystoscopy had positive FISH assays. The study results demonstrate that in patients with negative urine cytology results, although the cases with positive cystoscopy could be detected by FISH assay, FISH analysis was unnecessary to detect obvious tumors, because the tumors was easy to be revealed by cystoscopy and subsequent biopsies, but it was beneficial in those patients with negative cystoscopy findings. Previous study reported that if considering the low incidence of cancer (1.1%) in the patients with negative urine cytology and normal cystoscopy and the high cost (up to $800/FISH assay) of FISH assay, FISH analysis might not be cost effective assay.[15] Likewise, the FISH probes (GP Medical Technologies, Ltd, Beijing, China) in the current study are expensive and the patient needs to spend about $400 for FISH assay. Hence, the current high cost for FISH assay seems to hinder its clinical application for diagnosis and surveillance of BUC. In addition, in another study it has been reported that FISH assay is beneficial in patients with atypical urine cytology and negative cystoscopy,[19] and FISH assay is more helpful in patients with atypical urine cytology than negative urine cytology. The possible explanation was that, patients with atypical urine cytology might be more susceptible to have abnormal chromosomes than negative cytology.[15]

In the present study, FISH assays missed 22 patients with confirmed BUC; 16 (72.7%) of those patients had low grade tumors and 20 (90.9%) had non-muscle invasive tumors, which is consistent with previous report.[11] One explanation is that, low grade lesions do not actively shed carcinoma cells into the bladder lumen or, alternatively, such lesions do not exhibit the chromosomal changes that are detected by FISH assay.[20] Furthermore, the majority of the 22 patients with FISH-negative results also had negative urine cytology test results, and surprisingly, two cases who had negative FISH analysis were identified using urine cytology. One another possible explanation is that there were sufficient malignant cells in the specimen for urine cytology and insufficient malignant cells in the residual specimen for FISH assays after cytology processing; the other explanation is that malignant cells with morphologically abnormal unclear for urine cytology, did not have the most frequently altered chromosomes (3, 7, 9, and 17) for FISH analysis, but had another altered chromosomes.[21]

FISH assay as an independent prognostic factor on a chromosomal basis in non-muscle invasive BUC can predict recurrence or progression of tumor.[22] In our study, 69.2% of patients with positive FISH assay developed recurrent BUC during follow-up period, while only 6.7% of FISH-negative patients had recurrence. This finding agrees with previous reports.[20,23] It seems that the false-positive FISH results actually have not been false, but have preceded a tumor that could be detected by cystoscopy. Our results suggest that FISH assay may be useful as an early predictor of tumor recurrence. FISH-positive result may signal a high risk, while FISH-negative result signal a low risk for early recurrence and this finding may reduce frequency of follow-up cystoscopy. However, some studies reported that FISH assay did not provide any additional information and there was no evidence that positive FISH assay provided any “expectation” of future recurrence.[24,25] So far, a more aggressive workup of patients with positive FISH assay and negative cystoscopy is not currently justified.[26] As such, further studies are necessary to determine whether the FISH assay can provide additional information for evaluating the risk for recurrence or not. In the control group, among the three patients with positive FISH assay, no one was diagnosed with BUC or upper tract UC during follow-up period. This might suggest that chromosomal instability is not absolutely specific for bladder carcinoma,[27] and these could also be observed with reactive states, especially in the area of superficial urothelial cells.[28] The false-positive FISH results reduce the specificity of this assay.

According to one prior study, the karyotypic profile of the upper urinary tract UC is similar to that of the BUC.[29] Some researchers used FISH assay as an adjunct tool in managing upper urinary tract UC and reported that the cases could be diagnosed with a high specificity, using FISH assay.[18] In addition, it has been reported that FISH analysis is significantly better than urine cytology for diagnosing upper urinary tract UC.[30] In present study, among four patients with pathologically confirmed ureter UC, three patients were FISH-positive, while only one had positive urine cytology results. Furthermore, our study also included three patients who had bladder adenocarcinoma. As reported previously,[20] obvious amplifications of CEP 3, 7 and 17 have been found in all patients’ urine shedding cells and the patients were FISH-positive. It is difficult to assess the clinical implications of FISH assays in cases of upper urinary tract UC and bladder non-urothelial carcinoma, because the sample size of this study is low.

In our study, 12 patients were excluded due to uninformative FISH assays. The uninformative result occurs when there are insufficient shedding cells and presence

of massive granulocytes and bacteria in the voided urine. It was extremely difficult for the examiner to distinguish granulocytes from shedding cells during interpretation. In addition, bacterial signals that adhered to the shedding cells made accurate signal detection impossible. This is a limitation of the assay itself.

Our study has several limitations. Firstly, we did not evaluate the utility of FISH assay for detection of BUC in the patients with atypical urine cytology and negative or equivocal cystoscopy. Generally, FISH assay should not be used in patients with positive urine cytology or cystoscopy; FISH results are crucial for the clinician to detect new or recurrent bladder carcinoma in patients with atypical urine cytology and negative or equivocal cystoscopy results. Secondly, small sample size precluded evaluating the specificity of FISH and urine cytology. Finally, in addition to relatively small sample size the follow-up period for surveillance of BUC was also short.

CONCLUSION

Our results suggest that FISH assay can be used as a non-invasive diagnostic tool for patients suspected of having new BUC. In addition, FISH analysis may provide important prognostic information to better define the individual risk for BUC recurrence. However, large scale prospective studies are needed to better determine if negative FISH results can reduce the frequency of cystoscopy during follow-up period as well as to evaluate the prognostic role of FISH assay.

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CONFLICT OF INTEREST

None declared.

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