Targeting bladder tumor cells in voided urine of Chinese patients with FITC-CSNRDARRC peptide ligand

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Objective: To study the practicality of the FITC-CSNRDARRC peptide ligand (containing the Cys–Ser–Asn–Arg–Asp–Ala–Arg–Arg–Cys nonapeptide) in diagnosing and monitoring bladder tumors.

Materials and methods: Between March 2011 and September 2011, 80 consecutive patients with radiographic abnormalities, localizing hematuria, other symptoms, or signs were studied using the FITC-CSNRDARRC ligand, urinary cytology (UC), and fluorescence in situ hybridization (FISH). The sensitivity and specificity of these three technologies were determined and compared. Cystoscopy and tissue biopsy were taken as the “gold standards” for bladder tumor diagnosis in this study.

Results: Twenty-nine out of 80 patients were diagnosed with a bladder tumor via histopathological examination. The FITC-CSNRDARRC ligand was positive in 23 out of 29 bladder tumor patients and produced false negatives in six (20.69%) patients. The UC was positive in six out of 29 bladder tumor patients and produced false negatives in 23 (79.31%) patients. The FISH was positive in 21 out of 29 bladder tumor patients and produced false negatives in eight (27.59%) patients. The overall sensitivity as verified by the FITC-CSNRDARRC ligand was much higher than in UC (79.31% versus 20.69%, \( P < 0.001 \)) and was slightly higher than in FISH (79.31% versus 72.41%, \( P = 0.625 \)). The sensitivity of FISH was significantly higher than that of UC (72.41% versus 20.69%, \( P < 0.001 \)). Sensitivities of the FITC-CSNRDARRC ligand and UC by grade were 58.33% versus 8.3% for low-grade (LG) tumors (\( P = 0.031 \)) and 94.12% versus 29.41% for high-grade (HG) tumors (\( P = 0.003 \)), respectively. The advantage was maintained in terms of the detection of invasive tumors between the FITC-CSNRDARRC ligand and UC (90.48% versus 23.81%, \( P = 0.001 \)) as well as between FISH and UC (85.71% versus 23.81%, \( P = 0.003 \)). The specificities for the FITC-CSNRDARRC ligand, UC, and FISH were 100%.

Conclusion: Results show that the FITC-CSNRDARRC ligand is a promising noninvasive tool for diagnosis and surveillance in patients suspected of having a new bladder tumor.

Keywords: bladder tumor, tumor-targeting, FITC-CSNRDARRC ligand, fluorescent probe

Bladder tumors, accounting for 3.3% of all malignancies, are the ninth most common type of tumor worldwide.¹,² Bladder tumor patients are at high risk of recurrence and 7%–40% of patients will develop invasive tumors.³–⁵ The incidence of bladder tumors is also very high in China and has increased year on year. The main concern for doctors is in discerning which techniques should be used to diagnose bladder tumors in their early stages in order to improve the quality of the patients’ life. Early diagnosis and the monitoring of disease recurrence play a crucial role in the diagnosis of bladder tumors.
Optical imaging has been increasingly used to probe protein function and gene expression in live animals. Peptide targeting of tumors is a new technique used for the diagnosis of bladder tumors. The CSNRDARRC ligand is the most recently developed diagnostic probe for the detection of bladder cancer.

The CSNRDARRC sequence was selected as a synthetic peptide. In the study by Lee et al., the fluorescein-conjugated CSNRDARRC peptide selectively bound to human bladder tumor tissue, whereas only negligible binding to normal bladder tissue was observed. In our study, we made a further test of the feasibility of the peptide in detecting tumor cells in urine, with the peptide showing little binding to the urinary cells of patients with inflammation, or to healthy individuals’ cells.

Knowledge concerning the genetic changes in bladder tumor cells provides the basis for the development of genetic urine tests. FISH proved to be an effective method for identifying chromosomal aberrations. In 2000, Sokolova et al. developed what would become the first commercially available FISH probe set for bladder urothelial carcinoma detection in voided urine. The UroVysion FISH detection system (Abbott Molecular, DesPlaines, IL) received Food and Drug Administration (FDA) approval in 2001 for the detection of recurrent tumors in patients with a history of bladder cancer, while the use of the FISH technique is still in the clinical trial stage in China.

UC has a low sensitivity (<50%) but a high specificity (>90%), particularly for LG tumors. Compared with UC, alternative methods, such as the bladder tumor antigen (BTA) test, the urinary nuclear matrix protein 22 (NMP22), surviving (SV), and telomerase tests have become available FISH probe set for bladder urothelial carcinoma detection in voided urine. The UroVysion FISH detection system (Abbott Molecular, DesPlaines, IL) received Food and Drug Administration (FDA) approval in 2001 for the detection of recurrent tumors in patients with a history of bladder cancer, while the use of the FISH technique is still in the clinical trial stage in China.

Urinary cytology

Urine preparation for FISH

A hypotonic solution of potassium chloride (0.075M) was preheated in a constant temperature bath at 37°C for at least 30 minutes. For each sample, 50 mL of voided urine was centrifuged at 1300 rpm for 10 minutes at room temperature and then the supernatant was discarded. After pretreatment with 10% formaldehyde fixative, approximately 30 uL of the cell pel ight was dropped onto a standard glass slide. The glass slide was heated for 30 minutes at 56°C. After Papanicolaou or hematoxylin staining, the cell pel ight was examined by microscope.

FISH analysis

Cystoscopy and tissue biopsy were taken as the “gold standards” for bladder tumor diagnosis in this study.

Materials and methods

Voided urine specimens from 80 consecutive patients (mean age, 67.27 years; standard deviation [SD], 3.25 years; range, 41–88 years; males, 58 cases; females, 22 cases) with radiographic abnormalities, localizing hematuria, other symptoms, or signs were obtained between March 2011 and September 2011.

Urinary cytology

For each sample, 50 mL of voided urine was centrifuged at 1300 rpm for 10 minutes at room temperature and then the supernatant was discarded. After pretreatment with 10% formaldehyde fixative, approximately 30 uL of the cell pellet was dropped onto a standard glass slide. The glass slide was heated for 30 minutes at 56°C. After Papanicolaou or hematoxylin staining, the cell pellet was examined by microscope.
followed by washing in 0.4 × SSC/0.3% Nonidet (NP40) and 2 × SSC. After hybridization, the nuclei counterstaining was achieved with 4’,6-diamidino-2-phenylindole (DAPI). Slides were scored for hybridization signals on a cell-by-cell basis using a fluorescence microscope.

In every case, the severity of the genetic alteration was defined as the percentage of cells showing cytogenetic abnormalities greater than the cut-off values (ie, the number of cells that showed a gain for at least two of the chromosomes 3, 7, and 17, or that showed complete deletion of the p16 (9p21) locus divided by the total enumerated cells, with the quotient multiplied by 100).

**FITC-CSNRDARRC ligand**

The procedure for the urine preparation for the FITC-CSNRDARRC ligand was the same as that of FISH.

The 15 mg/L solution of FITC-CSNRDARRC ligand in PBS was added to the glass slide and then it was incubated at 4°C for 1 hour. After being washed in PBS and air-dried, the nuclei counterstaining was achieved with DAPI. Slides were scored for hybridization signals on a cell-by-cell basis using a fluorescence microscope.

**Statistical analysis**

The sensitivities of the FITC-CSNRDARRC ligand, UC, and FISH were determined for patients with biopsy-proven urothelial carcinoma in the bladder, and the specificities were calculated for patients with no histological confirmation of urothelial carcinoma or with negative cystoscopy findings. McNemar’s test was used to determine the statistical difference among the use of the three techniques (FITC-CSNRDARRC ligand, UC, and FISH). A P-value of <0.05 was considered to indicate statistical significance. The SPSS 15.0 software package (SPSS, Chicago, IL) was used for statistical analyses.

**Results**

A total of 80 voided urine specimens were analyzed in this study: 58 from men and 22 from women. Twenty-nine patients were diagnosed as having a bladder tumor, of which two had pTa-LG disease, three had pTa-HG disease, two had pT1-LG disease, one had pT1-HG disease, three had pT2-LG disease, six had pT2-HG disease, five had pT3-LG disease, four had pT3-HG disease, and three had pT4-HG disease.

**FITC-CSNRDARRC ligand**

The FITC-CSNRDARRC ligand was positive in 23 out of 29 bladder tumor patients and produced false negatives in six (20.69%) patients. The sensitivities and specificities of the FITC-CSNRDARRC ligand were 79.31% and 100%, respectively (Table 1).

The FITC-CSNRDARRC ligand was produced by TASH Biotechnology Co, Ltd (Shanghai, China). The CSNRDARRC peptide selectively bound to the nuclei of bladder tumor cells. The cells were determined as being positive if there were fluorescent probe signals in the nuclei (Figure 1).

**Urinary cytology**

Of the 29 bladder tumor patients, six patients had a positive UC result and 23 had a false-negative UC result (79.31%). The sensitivities and specificities of the UC were 20.69% and 100%, respectively (Table 1).

Results were assessed as positive only in cases where malignancy was detected (Figure 2C). Suspicious and equivocal cases were assessed as negative for the calculations of sensitivity and specificity.

**FISH**

The FISH showed positive results in 21 out of 29 bladder tumor patients and produced false negatives in eight (27.59%) cases. The sensitivities and specificities of the FISH were 72.41% and 100%, respectively (Table 1).

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**Table 1** Detailed comparison of FITC-CSNRDARRC ligand, UC and FISH for the detection of bladder tumor in relation to tumor stages and tumor grades

<table>
<thead>
<tr>
<th>Tumors</th>
<th>UC</th>
<th>FISH</th>
<th>FITC-CSNRDARRC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/N</td>
<td>n/N</td>
<td>n/N</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>1/12</td>
<td>6/12</td>
<td>7/12</td>
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<tr>
<td>High</td>
<td>5/17</td>
<td>15/17</td>
<td>16/17</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTa + pT1</td>
<td>1/8</td>
<td>3/8</td>
<td>4/8</td>
</tr>
<tr>
<td>pT2 – pT4</td>
<td>5/21</td>
<td>18/21</td>
<td>19/21</td>
</tr>
<tr>
<td>Overall sensitivity</td>
<td>6/29</td>
<td>21/29</td>
<td>23/29</td>
</tr>
<tr>
<td>Overall specificity</td>
<td>51/51</td>
<td>51/51</td>
<td>51/51</td>
</tr>
</tbody>
</table>

**Abbreviations:** FISH, fluorescence in situ hybridization; UC, urinary cytology.
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Probes specific for the centromeres of chromosomes 3, 7, 17, and for the $p16$ locus (GP Medical Technologies, Ltd, Beijing, China) were used in this study (Figure 3). Two DNA probes were mixed together as a set double-target FISH and paired as follows: chromosome 3 and $p16$ (rhodamine), chromosome 7 and 17 (FITC).

Figure 1 The FITC-CSNRDARRC ligand. (A and C) The nuclei counterstaining was achieved with DAPI. The light colored area in (B) is the nuclei. (D) DAPI was not used in the nuclei.

Notes: The CSNRDARRC peptide is selectively bound to the nuclei of bladder tumor cells. The bright dot pointed at by the arrows indicate where the peptides bind. Magnification $\times$1000 (A–D).

Abbreviations: DAPI, 4', 6-diamidino-2-phenylindole.

Statistical analysis

There is a significant difference in bladder tumor diagnosis in terms of the sensitivities of the FITC-CSNRDARRC ligand and UC (79.31% versus 20.69%, $P < 0.001$) (Tables 1 and 3), while no significant differences were found between the FITC-CSNRDARRC ligand and FISH (79.31% versus 72.41%, $P = 0.625$) (Tables 1 and 3). A significant difference was found between the sensitivities of FISH and UC (72.41% versus 20.69%, $P < 0.001$) (Tables 1 and 3). Meanwhile, the overall specificity for the FITC-CSNRDARRC ligand, UC, and FISH was 100%, which was not statistically significant.

Sensitivities of the FITC-CSNRDARRC ligand and UC by grade were 58.33% versus 8.3% for LG ($P = 0.031$) and 94.12% versus 29.41% for HG ($P = 0.003$) (Tables 1 and 3), respectively, whereas the sensitivities of FISH and UC by grade were 41.67% versus 8.3% for LG ($P = 0.063$) (Tables 1 and 3) and 88.24% versus 29.41% for HG tumors ($P = 0.006$) (Tables 1 and 3), respectively. Moreover, the advantage is maintained for invasive tumors between the FITC-CSNRDARRC ligand and UC (90.48% versus 23.81%, $P = 0.001$) (Tables 1 and 3) as well as between FISH and UC (85.71% versus 23.81%, $P = 0.003$) (Tables 1 and 3).

Discussion

In the present study, we compared the performances of UC, FISH, and the FITC-CSNRDARRC ligand in the detection of bladder tumor cells in voided urine.

The high sensitivity obtained with the use of the FITC-CSNRDARRC ligand in the 29 patients with bladder tumors was 79.31%, whereas with FISH it was 72.41%, compared with UC at only a 20.69% (both $P < 0.001$) detection rate. No significant difference was found between the FITC-CSNRDARRC ligand and FISH ($P = 0.625$). As previously
Table 2 The predictive values of three methods

<table>
<thead>
<tr>
<th>Classification</th>
<th>UC (%)</th>
<th>FISH (%)</th>
<th>FITC-CSNRDARRC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPV</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>NPV</td>
<td>68.92</td>
<td>86.44</td>
<td>89.47</td>
</tr>
</tbody>
</table>

Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

Table 3 Statistical analysis obtained by FITC-CSNRDARRC ligand, UC and FISH for the detection of bladder tumor

<table>
<thead>
<tr>
<th>Tumor grades</th>
<th>Overall sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-value* (2-sided)</td>
<td>Tumor stages</td>
</tr>
<tr>
<td>UC vs FISH</td>
<td>0.063</td>
</tr>
<tr>
<td>UC vs FITC-CSNRDARRC</td>
<td>0.031</td>
</tr>
<tr>
<td>FISH vs FITC-CSNRDARRC</td>
<td>l</td>
</tr>
</tbody>
</table>

Note: *Statistically significant difference (P < 0.05), McNemar test.
Abbreviation: FISH, fluorescence in situ hybridization; UC urinary cytology.
FITC-CSNRDARRC ligand will be a practical method for the detection and follow-up of bladder tumors and may aid in establishing the original diagnosis of the disease.

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Disclosure
The authors report no conflicts of interest in this work.

References