Original Article

Urinary cytology with acridine orange fluorescence is highly valuable for predicting high-grade upper urinary tract urothelial carcinoma

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Abstract: Objectives: To evaluate the clinical value of acridine orange fluorescent staining in urinary cytology for the diagnosis of upper urinary tract urothelial carcinoma. Methods and materials: A retrospective analysis was conducted with 510 cases of upper urinary tract urothelial carcinoma (UTUC) in terms of the results of acridine orange fluorescence (AO-F) staining of the exfoliated cells in urine. The percentage of positive AO-F result and the positive predictive value of AO-F for high-grade and muscle invasive urothelial carcinoma were calculated and analyzed in terms of clinical characteristics. Results: The overall percentage of positive AO-F result was 49% in the 510 patients, 54.1% for males and 40.6% for females. AO-F was positive in 51.9% of the patients with hematuria and 36.2% of the patients without hematuria. AO-F was positive in 56.4% of the patients with renal pelvis carcinoma and 42.8% of the patients with ureteral cancer; in 44.6% of the patients with non-muscle invasive carcinoma and 53.5% of the patients with muscle-invasive carcinoma. AO-F was positive in 26.8% of the cases with low-grade carcinoma and 55.3% of the patients with high-grade carcinoma. The positive predictive value of AO-F was 88% for high-grade cancer, and only 53.6% for muscle invasive carcinoma. Conclusions: Acridine orange fluorescence microscopy cannot increase the sensitivity of urine exfoliative cytology in the diagnosis of UTUC. It may be used as a predictor of high-grade UTUC. Acridine orange fluorescence microscopy in urinary cytodiagnosis does not show high value in predicting muscle invasive UTUC.

Keywords: Urothelial carcinoma, upper urinary tract, acridine orange fluorescence, cytodiagnosis

Introduction

The definitive diagnosis of upper urinary tract tumors before operation has been challenging in clinical practice. The specificity of urine exfoliative cytology is close to 100%. However, the low sensitivity in diagnosis of upper urinary tract urothelial carcinoma (UTUC) is a major reason preventing its extensive use in clinical setting. This retrospective analysis reviewed the results of acridine orange fluorescent staining of the exfoliated cells in urine for diagnosis of 510 cases of UTUC in our hospital, and evaluated the value of acridine orange fluorescence (AO-F) in the diagnosis of UTUC.

Materials and methods

A total of 524 patients with upper urinary tract tumor were treated in our hospital from July 2005 to October 2012. The patients were excluded if they had tumor of renal pelvis complicated with tumor of ureter, benign tumor of upper urinary tract, non-urothelial epithelial cancer of upper urinary tract, or no result of urinary cytology was available, or no tissue sample was available for pathological examination. The clinical data of 510 UTUC cases were included in this retrospective analysis. This study has been approved by institutional review board of second hospital of Tianjin medical university, because this is a retrospectively study, we got the data of patients from medical record library and we analysis anonymously of data, so the informed consent was waived.

Pathological diagnosis was confirmed for all patients following radical operation or partial ureterectomy. Pathological staging was designated as non-muscle invasive carcinoma (pT1a,
AO-F can predict high-grade UTUC

$pT_1$) or muscle invasive carcinoma ($\geq pT_2$) according to the TNM staging released by Union International Control le Cancer (UICC) in 2009 [1]. Histological grade was rated as low or high grade urothelial carcinoma according to the grading system recommended by World Health Organization (WHO) in 2004 [2].

The exfoliated cells in urine were examined by urinary cytology with acridine orange fluorescent staining. Specifically, fresh urine sample about 30-40 ml was collected by patient from the mid-stream urine of his/her second urination since getting up in the morning. The urine sample was centrifuged. The supernatant was discarded. Fixative solution was added to resuspend the sediments. The suspension was loaded to a glass slide and dried. The slide was immersed in acridine orange staining solution for 3 minutes, and rinsed with phosphate-buffered saline (PBS). Then the slide was soaked in 0.1% calcium chloride solution for 3 minutes. The slide was washed with PBS once again. Cover glass was mounted for observation under a fluorescence microscope to observe and read the result.

No uniform diagnostic criteria were available regarding acridine orange fluorescent staining. Generally, normal epithelial cells and inflammatory cells show bright green nucleus and greyish green cytoplasm under fluorescence microscope (Figure 1), which is defined as negative result. Malignant tumor cells demonstrate yellow or green nucleus and flame-like reddish orange fluorescence of cytoplasm with unique appearance of dyskaryosis and larger karyoplasms ratio under a fluorescence microscope (x200), which is defined as positive result. Other cells suspected of tumor are defined as indeterminate.

Chi-square test was used to compare the percentage of positive diagnosis between groups in terms of urine exfoliative cytology. The difference was defined as statistically significant when $P < 0.05$. Data analysis was performed with SPSS software version 17.0.

**Results**

A total of 510 patients were enrolled. The mean age was 66.8 (range 35-92) years old. AO-F was positive in 250 (49%) patients, 54.1% (172/318) in males and 40.6% (78/192) in females. The difference between males and females was statistically significant ($P < 0.05$). Clinical hematuria was reported in 416 patients, and 51.9% of them showed positive AO-F. About 36.2% of the remaining 94 patients without hematuria had positive AO-F ($P < 0.05$). Clinical hematuria was reported in 416 patients, and 51.9% of them showed positive AO-F. About 36.2% of the remaining 94 patients without hematuria had positive AO-F ($P < 0.05$). AO-F was positive in 56.4% of the 234 patients with renal pelvis carcinoma, and in 42.8% of the 276 patients with ureteral cancer. The difference was statistically significant ($P < 0.05$). AO-F was positive in 44.6% of the 260 patients with non-muscle invasive carcinoma and in 53.5% of the 250 patients with muscle-invasive carcinoma ($P < 0.05$). AO-F showed positive result in 26.8% of the 112 patients with low-grade cancer and 55.3% of the 398 patients with high-grade cancer ($P < 0.05$). The detailed data are provided in Table 1.
AO-F can predict high-grade UTUC

Post-surgical pathological examination confirmed that of the 250 patients with positive result of urinary cytology, 88% (220/250) were high-grade urothelial carcinoma, 12% (30/250) were low-grade urothelial carcinoma; 53.6% (134/250) were muscle-invasive carcinoma and 46.4% (116/250) were non-muscle invasive carcinoma. The positive rate and positive predictive value (PPV) of acridine orange fluorescence in diagnosis of high-grade and muscle invasive upper urinary tract urothelial carcinoma are shown in Table 2.

**Discussion**

UTUC accounts for 5-10% of all urinary tract epithelial tumors, and about 10% of all renal tumors [3]. The diagnosis of upper urinary tumors is mainly dependent on symptoms and imaging. CT urography has replaced intravenous urography as the golden standard in diagnosis of upper urinary tract tumors [4]. Urothelial carcinoma of both bladder and upper urinary tract has histological homology [5]. For this reason, urinary cytodiagnosis is also applicable to the diagnosis of upper urinary tract tumors. The sensitivity of this method is up to 95% for bladder carcinoma, but not satisfactory in identifying the urothelial carcinoma of upper urinary tract.

Acridine orange is a fluorescent chrome yellow dye, which can bind to intracellular DNA and RNA in different ways to produce fluorescence of different colors. So the malignant cells can be differentiated from the benign ones based on the color change of fluorescence and cell morphology. The biochemical abnormality of tumor cells precedes the morphological change of cells. Therefore, urinary cytodiagnosis with acridine orange staining can improve the performance of early diagnosis of cancers. Our previous studies have found that acridine orange has a sensitivity of 78.05% in identifying bladder carcinoma [6], which is higher than that of the conventional hematoxylin and eosin staining or Feulgen staining.

However, after this retrospective analysis of 510 cases of UTUC, we found that the positive rate of acridine orange staining was only 49% in diagnosis of UTUC, 56.4% in diagnosis of renal pelvis carcinoma, and 42.8% in diagnosis of Table 1. Clinical data and acridine orange fluorescence urinary cytodiagnosis of the 510 cases of upper urinary tract urothelial carcinoma

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Positive (n)</th>
<th>Negative (n)</th>
<th>Equivocal (n)</th>
<th>Total (n)</th>
<th>Positive rate (%) (95% CI)</th>
<th>X²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>172</td>
<td>119</td>
<td>27</td>
<td>318</td>
<td>54.1 (48.6 - 59.6)</td>
<td>8.68</td>
<td>0.003</td>
</tr>
<tr>
<td>Female</td>
<td>78</td>
<td>91</td>
<td>23</td>
<td>192</td>
<td>40.6 (33.7 - 47.5)</td>
<td></td>
<td></td>
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<td>Diagnosis</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Renal pelvis carcinoma</td>
<td>132</td>
<td>88</td>
<td>14</td>
<td>234</td>
<td>56.4 (50.0 - 62.8)</td>
<td>9.45</td>
<td>0.002</td>
</tr>
<tr>
<td>Ureteral cancer</td>
<td>118</td>
<td>122</td>
<td>36</td>
<td>276</td>
<td>42.8 (40.0 - 48.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematuria</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Yes</td>
<td>216</td>
<td>172</td>
<td>28</td>
<td>416</td>
<td>51.9 (47.1 - 56.7)</td>
<td>7.61</td>
<td>0.006</td>
</tr>
<tr>
<td>No</td>
<td>34</td>
<td>38</td>
<td>22</td>
<td>94</td>
<td>36.2 (26.5 - 45.9)</td>
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<tr>
<td>Tumor staging</td>
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<td></td>
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</tr>
<tr>
<td>Non-muscle invasion (T₁, T₂)</td>
<td>116</td>
<td>120</td>
<td>24</td>
<td>260</td>
<td>44.6 (38.6 - 50.6)</td>
<td>4.12</td>
<td>0.042</td>
</tr>
<tr>
<td>Muscle invasion (≥ T₂)</td>
<td>134</td>
<td>90</td>
<td>26</td>
<td>250</td>
<td>53.5 (47.3 - 59.7)</td>
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<tr>
<td>Tumor grading</td>
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<tr>
<td>Low-grade</td>
<td>30</td>
<td>70</td>
<td>12</td>
<td>112</td>
<td>26.8 (18.6 - 35.0)</td>
<td>28.39</td>
<td>0.000</td>
</tr>
<tr>
<td>High-grade</td>
<td>220</td>
<td>140</td>
<td>38</td>
<td>398</td>
<td>55.3 (50.4 - 60.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>250</td>
<td>210</td>
<td>50</td>
<td>510</td>
<td>49.0 (44.7 - 53.3)</td>
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</tbody>
</table>

**Table 2.** Positive rate and positive predictive value of acridine orange fluorescence in diagnosis of high-grade and muscle invasive upper urinary tract urothelial carcinoma

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Positive rate (%) (95% CI)</th>
<th>Positive predictive value (%) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-grade UTUC</td>
<td>55.3 (50.4 - 60.2)</td>
<td>88.0 (83.7 - 92.3)</td>
</tr>
<tr>
<td>Muscle invasive UTUC</td>
<td>53.5 (47.3 - 59.7)</td>
<td>53.6 (47.0 - 60.2)</td>
</tr>
</tbody>
</table>
AO-F can predict high-grade UTUC

ureteral cancer. These figures are not significantly higher than the previously reported sensitivity of urinary cytology for UTUC between 43% and 78% [7, 8]. Hence, urinary cytodiagnostics with AO-F cannot improve the positive rate in identifying UTUC. The positive rate of cytodiagnostics is closely related to the number of cells. It is therefore presumed that the following factors may contribute to such low sensitivity. Firstly, UTUC is usually solitary and smaller in size. Secondly, the exfoliated cells from upper urinary tract are readily degraded in the bladder. Thirdly, UTUC is less likely to shed cells compared to bladder carcinoma [9]. In our experience, use of the urine sample collected from the second urination after getting up in the morning can avoid the potential cell lysis caused by the overnight urine. Furthermore, urinary cytological test should be repeated for three times to increase the positive rate. More additional cytological tests are appropriate if the result is equivocal.

The standard surgical approach to UTUC is radical resection of kidney, ureter and bladder cuff [10]. At present time, it is considered that the conservative therapies such as endoscopic tumor resection are also appropriate for the patients with renal insufficiency, solitary kidney and selected patients with early stage, low-grade disease [11, 12]. However, no method is available to effectively tell the accurate stage and grade of carcinoma before operation. This is one of the major causes leading to disease progression and relapse after conservative therapy and affecting the outcome.

Locoregional recurrence is highly prevalent after endoscopic resection of low-grade tumor in clinical practice. This fact makes us think whether a patient is accurately diagnosed in terms of carcinoma staging and grading before endoscopic resection. Jamie Messer et al [9] reported a retrospective analysis of 326 cases of upper-tract urothelial carcinoma. They found that urine cytology alone could not accurately predict the stage or grade of carcinoma. Ureteroscopic biopsy is currently considered as the golden standard for diagnosis of UTUC. Keeley et al [13] stated that ureteroscopic biopsy and post-surgical pathology were highly consistent in cancer grading. However, the tissue sample obtained by ureteroscopic biopsy is too small in size. More than a quarter of the patients with upper urinary tract tumor cannot be diagnosed appropriately [14]. Additionally, the histological grade varies with the site of the tumor due to the heterogeneous nature of carcinoma, which may lead to the missed diagnosis of high-grade disease [15].

Renshaw [7] and Williams et al [8] reported that the accuracy of identifying high-stage and high-grade urothelial carcinoma was improved by combining urine cytology with ureteroscopic biopsy. But Julia Straub et al [15] considered that the accuracy of urine cytology combined with ureteroscopic biopsy is still limited in predicting the preoperative histological grade of UTUC.

In our study, of the 250 patients with positive result of acridine orange staining, 220 were high-grade urothelial carcinoma. The PPV was up to 88%. And 134 were muscle invasive carcinoma. The PPV was only 53.6%. We consider that urine cytodiagnostics with AO-F can well predict high-grade UTUC. But it cannot provide good performance in predicting muscle invasive UTUC. This may be due to the early nucleotide change within high-grade cancer cell and the presence of numerous polyploid cells, which increase the sensitivity of the cells to AO-F. Acridine orange staining provides diagnosis based on both the morphological change of cells and the different fluorescent stain of nucleotide, which increases the specificity in identifying high-grade carcinoma. However, the number of exfoliated cells does not increase with the depth of tumor infiltration. Therefore we think that positive AO-F can be taken as a predictor of high-grade carcinoma. More proactive radical therapy should be considered for the patients with positive AO-F to avoid disease progression and relapse resulted from conservative treatment such as endoscopic tumor resection. However, it is impossible to calculate the overall specificity as well as the positive and negative predictive values of AO-F due to its retrospective nature. Randomized controlled study is required with large sample to confirm the findings of this study.

Conclusion

AO-F cannot increase the sensitivity of urine cytology in diagnosis of upper urinary tract tumors. Urine cytology with AO-F has a high PPV for high-grade UTUC, so positive AO-F can be used as a predictor of high-grade UTUC.
AO-F can predict high-grade UTUC

Proactive radical treatment should be administered to the patients with positive AO-F. The conservative intracavitary therapy should be used cautiously because such a treatment may lead to high recurrence of cancer. The studies on diagnosis of UTUC by using tumor markers and new laboratory tests are under the way. It is believed that in the near future new laboratory tests will provide accurate preoperative staging and grading of carcinoma for the patients with upper urinary tract tumor. More and more patients will benefit from these new techniques.

Acknowledgements

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Disclosure of conflict of interest

We declare that we have no conflict of interest.

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