Prognostic Significance of Fluorescent In Situ Hybridisation in the Follow-up of Non-muscle-invasive Bladder Cancer

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Abstract. Aim: To evaluate the potential contribution of a fluorescent in situ hybridization (FISH) as prognostic indicator of the risk of recurrence or progression in patients undergoing follow-up for non-muscle-invasive bladder cancer (NMIBC). Patients and Methods: A total of 126 consecutive patients with a history of NMIBC being followed-up with urinary cytology and cystoscopy at a referral centre were studied. Patients with carcinoma in situ, or tumour stage higher than pT1 were excluded. A UroVysion FISH kit was used to detect four chromosomal abnormalities, specifically, locus 9p21, Ch 3, 7, and 17. Three FISH patterns were defined: negative; low-risk positive, i.e. positive staining for 9p21 and/or Ch3 abnormalities; and high-risk positive, i.e. positive staining for Ch7 and/or 17. Results: Overall 73 out of 126 patients (57.9%) had a positive urinary FISH test. After a median time of 14 months, 46 FISH-positive patients underwent recurrence (36.5%) and in 15 patients there was progression of disease (11.9%). Among positive patients, the low-risk category was found in 34, and the high-risk in 39. Low-risk FISH-positive patients had a higher rate of recurrence as compared to FISH-negative patients, with a hazard ratio (HR) of 1.6. The recurrence rate was even greater in patients with a high-risk positive test, with an HR of 1.9. The limitation of the study was that the impact of intravesical treatment was not assessed. Conclusion: The urinary FISH test can be used as an aid in predicting the risk of recurrence during follow-up of patients with history of NMIBC.

Tumour recurrence and progression are two clinically relevant events expected after the initial diagnosis of non-muscle-invasive bladder cancer (NMIBC), stage Ta-T1, low-, or high-grade disease. Recurrences and/or new occurrences are observed in up to 80% of cases, whereas progression of disease may vary from 5% to 50%, depending on grade, stage, size, and multiplicity of lesions (1, 2). With the purpose of assessing individual patient’s risk, look-up tables were recently made available (3). In order to detect subsequent episodes of disease, follow-up studies include cystoscopy and urinary cytopathology (UC). Cystoscopy is a relatively invasive procedure and although the specificity of UC is considered satisfactory, its sensitivity is reported to vary from 35 to 95%, dependent mainly on cellular grade (4). In order to improve on the sensitivity and specificity of UC and to reduce the need for cystoscopies, several alternative tests have been investigated (5). Urinary fluorescent in situ hybridization (FISH) has shown high sensitivity and specificity. Basically, FISH consists of DNA staining techniques with the capability of detecting multiple chromosomal abnormalities present in exfoliated cells (6). Urinary FISH is studied primarily in the detection of newly diagnosed, and recurrent transitional cell carcinoma (TCC) of the bladder and, to a lesser extent, as an indicator of prognosis (7, 8). Previous studies found no evidence to support the use of urinary FISH as a replacement for cystoscopy, and no improvement over UC (9, 10). We investigated the prognostic significance of the chromosomal abnormalities detected by FISH in urothelial cells from voided urine during the follow-up of patients with recurrent NMIBC. The findings were correlated with the subsequent course of the disease, namely, with the events of recurrence, and progression of grade, stage, or both. The primary aim was to assess whether a positive test was associated with an increased risk of recurrence, or progression, and the secondary aim was to assess the predictive potential of two different patterns of FISH positivity on such risk.

Patients and Methods

Patients with a previous diagnosis of Ta, T1, low-, or high-grade TCC undergoing cystoscopy and UC as follow-up examinations were included in the study. The presence of Tis, or a pathologic
stage higher than T1, constituted criteria for exclusion, whereas a previous exposure to intravesical chemo-, or immunotherapy was accepted. Urine samples were collected and submitted to FISH test.

**Follow-up.** Patients were followed with UC, and flexible cystoscopy at 3-month intervals. Urinary FISH was introduced beginning in May 2003.

**Urinary cytopathology.** Urine samples (≥50 ml) were sent for routine cytological examination with the thin prep technique, stained with standard Papanicolaou for cytologic grading according to the WHO criteria (11).

**UroVysion® FISH test.** The multi-target, multicolour UroVysion FISH assay, (Vysis/ABBOTT, Downers Grove, IL, USA) is composed of four directly labelled probes for the peri-centromeric region of chromosomes 3 (CEP 3), 7 (CEP 7), 17 (CEP 17), and to band 9p21 locus (LSI 9p21). The probes were labelled with different fluorescent dyes (spectrums red, green, aqua and gold).

**Enumeration of FISH signals.** All cases were evaluated without knowledge of UC or cystoscopy findings. The slides were scored for hybridization signals on a cell-by-cell basis using an Olympus fluorescence microscope (Provis AX 70; Olympus, Milan, Italy) with a filter set including (DAPI) single bandpass (DAPI counterstain), aqua (chromosome 17), gold single (9p21 locus), red (chromosome 3) and green (chromosome 7).

Overlapping cells and cells with indistinct or blurry signals were not scored. Chromosome 3 in the UroVysion assay may frequently present with ‘split signals’; these signals were not recorded as positive results in the FISH analysis and care was taken not to interpret split signals as two signals.

**Defining the cut-off for a positive FISH result.** The criteria used by previous investigators for defining FISH-positivity differ and no universally accepted criteria currently exist. In our study, we adopted the criteria described by the most extensive series published to date (12) and consistent with those suggested by the manufacturer. Normal cells contain two copies of each of the DNA targets, while tumour cells may show a different pattern, with losses or gains of signals. Enumeration of the signals was carried out on 25 target cells. A urothelial cell was considered abnormal by FISH if it showed three or more copies of any of the signals for chromosomes 3, 7, 17 and the 9p21 locus, or if there was heterozygous or homozygous loss of 9p21 (one copy or both copies lost).

A case was considered positive for malignancy if at least one of the following criteria were met: a) 4 or more cells with gain of more than 1 chromosome; b) 10 or more cells with gain of a single chromosome; c) 10 or more cells with homozygous loss of the 9p21 locus.

**Definition of low- and high-positivity.** Positive staining for locus 9p21, and for Ch 3, alone or combined was defined as low-positive, whereas positive staining for Ch 7 and Ch 17, alone or combined was defined as high-positive (7).

**Statistical analysis.** Categorical data were summarized as number (percentage) of individuals; continuous data were summarized as mean, standard deviation, median and range. Kaplan-Meier estimates (11) of the cumulative probability of recurrence or progression, defined as the time interval from baseline FISH results to the diagnosis of the event, were obtained for all patients and divided depending on baseline FISH results (negative vs. low-positive vs. high-positive). The Cox proportional hazards model (12) was used to estimate and to compare the risks of recurrence in terms of the hazard ratio (HR). Two-tailed probabilities and a p-value of 0.05 were used to define nominal statistical significance. All calculations were performed using STATA version 10 software (Stata Corp, College Station, TX, USA).

**Results**

From May 2003 to June 2008, a total of 431 patients underwent FISH testing at our hospital. A group of 152 consecutive patients undergoing follow-up with UC and cystoscopy for recurrent NMIBC at our Department were identified from the database and also submitted to FISH testing. The demographic data, the pathologic stage and grade as determined at last TURBT for patients included in the analysis are reported in Table I, along with the reasons for exclusion. The distribution of stage and grade with the results of the first FISH test, or baseline, are given in Table II.

After a median follow-up time of 14 (range: 1.5 to 59) months from the first FISH result, 46 out of 126 patients (36.5%) had experienced recurrence of disease. The median disease-free interval for the entire cohort was 3.2 years.

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**Table I. Demographic data and histology.**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Male/female (%)</th>
<th>Mean age, years (range)</th>
<th>Lost to follow-up</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>128/24 (78/22)</td>
<td>69.2 (44-86)</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

**Table II. Stage and grade distribution of patients according to FISH test result.**

<table>
<thead>
<tr>
<th>FISH test result</th>
<th>Patients (n=126)</th>
<th>Negative (n=53)</th>
<th>Low risk (n=34)</th>
<th>High risk (n=39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTaG1</td>
<td>43</td>
<td>25</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>pTaG2</td>
<td>32</td>
<td>14</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>pTaG3</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>pT1G1</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>pT1G2</td>
<td>35</td>
<td>12</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>pT1G3</td>
<td>8</td>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

**Histology**

- Tis: Transitional cell carcinoma.
- Muscle-invasive
- Upper urinary tract TCC
- Non-muscle-invasive
Progression of disease occurred in 15 out of 46 patients with recurrence (32.6%), or 15 of 126 (11.9%) overall. The remaining 80 patients (63.5%) were free from recurrence and progression. Overall, the FISH test was positive in 73 out of 126 patients (57.9%), and UC in 63 (50%). Among the 73 patients with a positive test, high-, and low-risk positivity were found in 39, and 34 patients, respectively. The Kaplan-Meier curves of the patients with negative, low-positive, and high-positive tests are plotted in Figure 1. Cumulative probabilities of event-free survival at 2 years follow-up in FISH-negative, low-risk positive, and in high-risk positive patients, were 0.76 (95% CI: 0.57-0.88), 0.64 (95% CI: 0.42-0.79) and 0.54 (95% CI: 0.37-0.69), respectively. The HR (Cox model) for disease recurrence, or progression, in patients with a low-positive compared to a negative test was 1.6 (95% CI: 0.73 to 3.46, \( p = 0.2 \)), whereas, in patients with a high-positive test, it was 1.9 (95% CI: 0.96 to 3.7, \( p = 0.1 \)).

In patients with baseline negative urinary FISH test result, the test turned positive before recurrence. Radical cystectomy was three times more frequent in patients with baseline positive FISH test (7 vs. 2). The outcome of the population under study with regards to recurrence and progression, and the need for further treatment (i.e. radical cystectomy) is presented in Figure 2.

**Discussion**

The value of urinary FISH in patients under follow-up for NMIBC has been investigated previously and a sensitivity of 30% to 79%, with a specificity of 68.5% to 95%, was found (5, 10). The relatively wide variability observed both in sensitivity and specificity is attributable to lack of uniformity in the criteria of execution of the test, and specifically, in the processing and scoring of positively stained cells over the total number of cells examined. The contribution of FISH as an aid to reduce the number of invasive cystoscopies has also been studied (10, 15). In comparison with standard follow-up, the test showed equal capability in detecting recurrences of NMIBC, but with a lower sensitivity; therefore, urinary FISH was deemed as unable to replace, or simply to reduce, the need for cystoscopy. It must be noted, however, that the low sensitivity is influenced by patient selection, that is, the
inclusion of patients with tumours of low malignant potential with limited or absent urinary shedding of cells; in addition, the lack of uniformity in the performance may impact on the results (5-10). Specifically, Moonen et al. evaluated the test in 105 patients and observed a sensitivity and specificity of 39.1% and 89.7%, respectively. The authors concluded that FISH test did not provide improvement over cytology in the follow-up of recurrent NMIBC (10). Of note, in this series both Cis and G3 tumours, that is, tumours with highly positive cytology rate, were also included. Gudjónsson et al., analysed 159 patients prospectively during surveillance of NMIBC (15). In a subgroup of 27 biopsy-proven recurrences the observed sensitivity for cystoscopy, cytology, and FISH was 93%, 22%, and 30%, respectively, with only 8 patients having a positive FISH test. However, the proportion of patients with tumours of low malignant potential and stage pTa tumours included in the analysis was 17 out of 27.

Pycha and co-workers, first used urinary FISH to characterize the behaviour of urothelial cancer and found that chromosomal aberrations including Ch 7 and 17 were associated with reduced progression-free survival (16). Patients with positively staining cells for Ch7 and 17 were categorized as high-risk, whereas those with positively staining cells for 9p21 and/or Ch 3, as low-risk. Subsequently, Mian et al. prospectively investigated patients under follow-up for superficial bladder cancer (7) and found recurrence in 27 out of 74 evaluable cases (36.8%), with a median time to recurrence of 16.7 months in 18 high-risk vs. 30.8 months in 9 low-risk patients. Interestingly, progression of disease was developed by 50% of high-risk patients, as opposed to 11% of low-risk patients.

Urinary FISH has the unique capability of detecting chromosomal abnormalities in exfoliated cells. The UroVysion multicolour multi-target assay is able to detect chromosomal abnormalities that can be found either in association with established urothelial cancer (Ch 7 and 17), or which mark an initial step in the carcinogenic process (9p21 and Ch 3), and may also represent one genetic fingerprint of urothelial ‘instability’. Data from previous trials (17, 18) have suggested that a positive urinary FISH test in the absence of visible tumour in the urinary tract should be interpreted as a consequence of unstable urothelium, harbouring the potential for malignant transformation. In fact, the FISH test has revealed the ability to detect tumour recurrence in almost one half of recurrent bladder cancer cases before clinical evidence at cystoscopy. Such ability is referred to as ‘anticipatory positivity’ (9). One clinical implication is that high-risk patients should benefit from attentive follow-up, whereas low-risk and negative patients may be equally safe with less frequent surveillance.

The primary aim of our study was to assess whether a positive test was associated with an increased risk of recurrence, or progression, and the secondary aim was to assess the predictive potential of two different patterns of positivity on such risk. We intended to correlate the events of recurrence and progression, that is, the natural history of the disease, with FISH findings. A positive FISH test correctly identified 32 out of 46 patients in whom recurrence of disease was histologically diagnosed (69.5%), and in addition, 11 out of 15 patients in whom a progression of stage, grade, or both was observed (73.3%). Cumulative probabilities of recurrence-free survival at 1, 2 and 3 years, showed a clear trend towards an increased risk from a negative test, to a low-, and to a high-positive. In addition, a positive FISH test identified patients for whom the risk of recurrence is increased as compared to negative patients, i.e. HR of 1.6. The risk was almost doubled in patients with a high-positive test, i.e. HR of 1.9 (Figure 1).

Finally, we do not advocate the use of the urinary FISH test as a routine part of the follow-up for all patients with NMIBC. It should be better restricted to intermediate-risk patients, which also allows for an optimization of the costs.

The study has some limitations, for example, the impact of intravesical therapy was not addressed. Intravesical therapy was initiated disregarding FISH results, adhering to the criteria of indications of the EAU guidelines on bladder cancer (19). In other words, a positive test did not influence the indication to treatment. Although we are unable to assess its impact on results this variable was equally present among the groups.
Another limitation is represented by the number of patients included in the study; however, we restricted the analysis to those patients who were undergoing complete follow-up tests (i.e. urine cytology, cystoscopy, and FISH test) at our Department only, by urologists and pathologists of internal institutional staff, with the intent of minimizing variability.

In conclusion, during follow-up a positive urinary FISH test represents an aid in identifying patients at risk of disease recurrence. In comparison to FISH test-negative patients, the low-positive patients had an increased risk of recurrence, with an HR of 1.6. The risk almost doubled in patients with the high-risk pattern. A positive urinary FISH test identified 32 out of 46 patients who subsequently developed recurrence, and 11 out of 14 among them who developed progression. In patients with a baseline negative FISH test, the test turned positive before recurrence. The need for further treatment, i.e., radical cystectomy, was three times greater in patients with a positive test at baseline. High-risk positive FISH identifies patients who need stringent follow-up, whereas low-risk, and FISH test-negative patients should be safe with less intense surveillance.

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References