Multiprobe Fluorescence in situ Hybridization (UroVysion) for the Detection of Urothelial Carcinoma – FISHing for the Right Catch

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Key Words

Cytology • Urine • Bladder cancer • UroVysion •
Fluorescence in situ hybridization • Chromosomes

Abstract

Urinary cytology has a well-established role in the detection and monitoring of urothelial carcinoma. The main strength of cytology is the high specificity for high-grade urothelial carcinoma and carcinoma in situ, but it has a low sensitivity for low-grade, non-invasive tumors. There are several other limitations of cytology. Cytology of the upper urinary tract and after intravesical therapy with bacillus Calmette-Guerin is notoriously difficult to interpret. In addition, there is a poorly defined but commonly used category of atypical cytology of uncertain significance. The UroVysion multiprobe fluorescence in situ hybridization has emerged as a helpful tool to address these limitations. It consists of fluorescently labeled DNA probes to detect increased copy numbers (polysomy) of the chromosomes 3, 7 and 17 and deletion of 9p21, the site of the *P16* tumor suppressor gene. Multiple studies have shown that fluorescence in situ hybridization in voided urine and washing specimens can help in patient management due to its superior sensitivity over cytology in different situations. It can be particularly useful to clarify equivocal cytological findings. However, some aspects remain to be further addressed including cost efficiency, optimal cut-off values and the true performance under real-life conditions.

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Introduction

Bladder cancer is the 6th most frequent cancer in developed countries [1]. Because of long-term survival and the need for lifelong routine monitoring and treatment, the cost per patient with bladder cancer from diagnosis to death is highest of all cancers [2]. The majority (70%) of bladder cancers are non-muscle invasive (stage pTa, pT1 and carcinoma in situ) [3]. Among these, 70% are low-grade non-invasive (pTa) tumors that often recur but almost never progress to life-threatening disease [4]. Combined cystoscopy and cytology are the gold standard for diagnosis and follow-up of urothelial carcinoma (UC) [5]. The high specificity for the diagnosis of high-grade UC is an undisputed strength of urinary cytology [6–8]. Cytology is particularly powerful in detecting non-visible carcinoma in situ. However, cytology performs weakly in low-grade UC due to an overlap of cytological features with benign urothelial cells. In addition, there is a commonly used but poorly defined category of atypical cytology that has questionable significance [9–11].

Development and progression of cancer are driven by genetic aberrations such as mutations and chromosome and gene copy number abnormalities. Fluorescence in situ hybridization (FISH) is a robust technique for the detection of chromosome copy number gains (polysomies), gains or amplifications and deletions of DNA loci, and translocations. FISH analysis of cytological specimens has become a standard method to identify chromo-

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somal aberrations for diagnostic, prognostic or predictive purposes [12–15]. The multiprobe FISH assay UroVysion (Abbott Molecular Inc., Des Plaines, Ill., USA) for the enumeration of chromosomes 3, 7 and 17 and the locus-specific identifier for the region at 9p21 was developed 10 years ago to overcome the diagnostic limitations of urinary cytology [16]. The 4 probes were selected since increased copy numbers (polysomies) of the chromosomes 3, 7 and 17 had been found to be particularly frequent in bladder cancer. Deletion of 9p21, the site of the tumor-suppressor gene *P16*, is also common and occurs early in the development of both papillary and flat urothelial neoplasia [17]. Subsequent progression is associated with chromosomal instability leading to aneuploidy with multiple chromosomal aberrations.

UroVysion FISH Analysis: Technical Aspects

Hybridization and Specimen Type

DNA FISH probes are labeled with fluorescent molecules and hybridize to pericentromeric regions of individual chromosomes or to selected DNA loci. The signals can then be enumerated under a microscope using appropriate fluorescent filters. UroVysion FISH is applicable to almost all types of cytological specimens such as conventional smears, cytospins or liquid-based specimens (e.g. ThinPrep® or SurePath®) regardless of fixation type (air-dried versus alcohol-based fixatives). FISH works equally well on unstained and Papanicolaou-stained specimens. We prefer hybridizing a Papanicolaou-stained cytospin specimen after confirming that the slide contains the urothelial cells of interest. For more details on the technical protocols of UroVysion testing, refer to the package insert and our previous methodology paper [18].

Scoring FISH Signals

The scanning technique to enrich abnormal cells rather than analyzing a high number of random cells is suggested for the FISH scoring. This is based on the observation that chromosomal polysomy is strongly associated with morphologic features of the nuclei [16, 19]. Twenty-five urothelial cells are selected based on nuclear abnormalities including enlargement, irregular borders and patchy nuclear staining with 4'-6-diamidino-2-phenylindole. According to the manufacturer's guidelines, a positive FISH result is defined as the presence of ≥ 4 morphologically abnormal cells with multiple gains (i.e. >2 signals of at least 2 of the 3 chromosomes 3, 7 or 17), or a loss

Fig. 1. FISH-positive result of a voided urine specimen: 3 tumor cell nuclei showing increased copy number (polysomy) of the chromosomes 3 (aqua, 4 signals), 7 (spectrum-red, 4–5 signals), but a normal copy number of chromosome 17 (spectrum-green, 2 signals). There is a loss (deletion) of 9p21 (spectrum-gold, 0–1 signals). $\times 1,000$.

of both copies (homozygous deletion) of 9p21 in $\geq 12/25$ cells (fig. 1). These criteria have proved valid in many studies [20–22]. However, further refinement is possible, and the criteria are evolving. According to Kipp et al. [23], quantitation of FISH abnormal cells by scoring at least 100 cells in every FISH-positive case can provide additional diagnostic information. They found that the percentage of abnormal cells was independently associated with recurrence and progression in patients with a history of non-invasive bladder cancer. The 9p21 probe also deserves special attention. In addition to homozygous deletion, there are low-grade UC with a heterozygous deletion of 9p21 as a sole manifestation of malignancy. These cases would be false negative by FISH using the stringent criteria of homozygous deletion in ≥ 12 cells. Finally, cytologically benign cells can be tetraploid due to transition through the S or G2 phase of the cell cycle or due to endoreplication. Therefore, we recommend to rate the sole presence of rare tetraploid cells (i.e. $\leq 10/25$) in bladder washings as negative [24, 25].

Reproducibility

The reproducibility of UroVysion FISH is poorly investigated. However, Brankley et al. [26] found that reproducibility seems to depend on the percentage of FISH

abnormal cells. Reproducibility among 7 cytotechnicians was 100% for 8 specimens with >10% abnormal cells, 93% for 2 negative specimens and lowest (78%) for 3 specimens with 1–10% abnormal cells. Thus, it is advisable to confirm FISH-positive results with few abnormal cells (e.g. 5/25 cells) by a second technician and interpret such a borderline result with caution.

Automation

Automation is not a prerequisite for successful UroVysion FISH scoring but can provide some advantages. The use of a relocation software that drives an automated stage for relocation of individual cells or cell groups is of most practical value and greatly facilitates routine FISH analysis. It allows saving the coordinates of cells of interests that were identified in the Papanicolaou-stained specimen, so that these specific cells can be analyzed for chromosomal aberrations after hybridization. Such relocation also facilitates the review and discussion of FISH findings with the microscope. Notably, this procedure eventually trains the morphology skills of the interpreters as it allows for a direct correlation between cell morphology and chromosomal aberrations. Several imaging systems have been developed for automatic counting of FISH signals. They generate image galleries that need to be edited at the computer screen for the final result. There are currently 2 US Food and Drug Administration-approved systems for automated UroVysion analysis including the Duet System (Bioview Ltd., Rehovoth, Israel, and Billarica, Mass., USA) [27, 28] and the Ikoniscope (Ikoni-sys Inc., New Haven, Conn., USA) [29]. These systems allow for permanent documentation and retrospective re-analysis of the FISH results. In addition, the Duet System considers both cytomorphology and FISH results for the final diagnosis. The interpretations by the Duet System were equivalent to manual interpretations and lead to modest time savings as compared to manual FISH analysis. Thus, automated FISH analysis systems might be an interesting option for laboratories with a high volume of FISH analyses [28].

Indications and Performance of UroVysion Testing in Urinary Cytology

The US Food and Drug Administration approved UroVysion testing of voided urine as an aid for initial diagnosis of bladder cancer in patients with hematuria and subsequent monitoring for tumor recurrence in patients previously diagnosed with bladder cancer [30, 31]. In rou-

Table 1. Indications for UroVysion FISH analysis in urinary cytology

Atypical urinary cytology
Control after intravesical BCG treatment
Upper urinary tract cytology
Surveillance after transurethral resection
Hematuria in patients with an increased risk of UC

tine practice, there are several additional situations in which FISH is frequently used (table 1). Two recent reviews and a meta-analysis provide detailed summaries on the performance of UroVysion FISH [22, 32, 33]. In the meta-analysis of Hajdinjak [22], the pooled sensitivity and specificity of FISH were 72 and 83%, as compared with 42 and 96% for cytology. UroVysion FISH has a sensitivity of 90–100% for the detection of invasive bladder cancer (pT1–4) and a specificity of >95% [33]. In the clinically far less relevant category of low-grade non-invasive bladder cancer, FISH increases the sensitivity of cytology from 25 to 60–75%.

Clarification of atypical (or equivocal) cytology has emerged as one of the most rewarding applications of urinary FISH analysis [23, 25, 34–37]. In their pivotal study, Skacel et al. [38] retrospectively evaluated the UroVysion FISH assay in 120 urine specimens and found a sensitivity of 100, 89 and 60% in patients with suspicious, atypical and negative cytology, respectively, while the overall specificity was 97%. A negative test result indicates the presence of benign cytological changes and helps to avoid unnecessary investigations such as repeat cystoscopy or other invasive procedures (fig. 2). A negative FISH result in case of a negative or atypical cytology does not exclude low-grade urothelial neoplasia, since up to 30% of these tumors are negative with the FISH assay. However, as outlined above, delayed diagnosis of these low-grade urothelial neoplasias is not a major problem, since progression to muscle-invasive cancer is very uncommon. Instead, a negative FISH result makes the diagnosis of a high-grade UC or carcinoma in situ unlikely [25]. Unfortunately, there is no clear morphological definition of the common diagnosis of ‘atypical urinary cytology’ [9–11, 39]. Whether a urinary cell is labeled as atypical or equivocal greatly depends on the training, experience and diagnostic confidence of individual observers. There is an obvious need for a uniform classification of urinary cytology as it exists for cervicovaginal cytology and thyroid cytology [40, 41]. A consensus definition of atypical/equivocal urinary cells would boost educational efforts and help to narrow

Fig. 2. Cytological atypia in an ureteral washing in favor of reactive changes. **a** Papanicolaou. $\times 400$. **b** FISH in the same field after automated relocation showing a negative result with 2 signals for each of the 4 probes in all cell nuclei. $\times 630$.

down indications for reflex FISH testing. To be meaningful, the atypia rate should be kept low.

Cytology after intravesical bacillus Calmette-Guerin (BCG) treatment of high-grade non-muscle invasive UC is notoriously difficult, since the reactive changes can appear most worrisome [25, 42, 43]. We found that patients with a positive post-BCG cytology or FISH result had a 3.2- and 3.8-times higher risk of recurrence, respectively, when compared to the patients with negative results [25]. Kipp et al. [19] and Mengual et al. [44] found a similar impact of FISH after BCG. However, these authors did not consider the impact of cytological diagnosis. In our study, high-grade cytology (G3) retained its high specificity after BCG treatment [25]. A positive FISH result was superior to cytology as a marker of relapse (hazard ratio = 6.2 and 1.4, respectively) when positive G3 cytology was excluded from the analysis. Two other recent studies confirmed that a positive FISH result in BCG-treated patients with a history of high-grade non-muscle-invasive UC predicts relapse independently of cytology and cystoscopy results [43, 45].

In patients under surveillance after a previously resected non-muscle-invasive UC, FISH at the time of a negative cystoscopy can help to better estimate the risk of recurrence [24, 30, 37, 46] although this was not confirmed in all studies [47, 48]. A positive FISH result at the time of a negative cystoscopy in patients with a sub-

sequent recurrence has been referred to as an 'anticipatory' positive FISH result. Notably, this 'anticipatory' quality is not restricted to FISH but can also be achieved by cytology [24]. It has been known for long by cytologists that a positive cytology bears important information irrespective of the cystoscopy or biopsy findings at the time of analysis [6, 49]. If confirmed in prospective trials, a combination of FISH and cytology might allow to better tailor the schedules of control cystoscopies according to the individual risk, and ultimately, reduce the number of cystoscopies in patients with a low-risk profile.

UC of the upper urinary tract (UUT) is rather uncommon and accounts for only 5% of urothelial tumors [50]. They are often diagnosed at a high stage, emphasizing the need for an early diagnosis. The sensitivity of cytology is often <50% in voided urines, while it is higher in ureteral and pelvic washings [51]. However, interpretation of UUT washing cytologies can be difficult due to instrumentation-related changes and lead to false-positive results. There are several published studies on the value of FISH for the detection of UC of the UUT [52–58]. In voided urines, sensitivity and specificity of FISH ranged from 54–86 to 78–100% as compared to cytology, which ranged from 24–40 to 96–100%. In a prospective study on UUT washing cytologies from 55 patients, the overall sensitivity was only 21% for cytology but 100% for FISH [55]. The

specificity was 97% for cytology and 90% for FISH. In summary, FISH appears to be an interesting tool to increase the sensitivity for the detection of UC of the UUT. However, the low specificities of 63 and 78% in 2 of the studies raise concerns about the possibility of false-positive results due to reactive conditions [53].

Pitfall: Chromosomal Abnormalities in Benign Cells

Chromosomal abnormalities are not restricted to malignancy but can also occur in benign cells. In particular, tetraploidy with a balanced duplication of the whole genome can prevail in non-neoplastic conditions of the bladder [59]. Most of these cells can easily be diagnosed as activated umbrella cells by those trained in cytology. Part of the limited specificity of FISH in some studies might be referable to the overinterpretation of benign tetraploid cells. Therefore, FISH should not be diagnosed as positive, solely based on cells with a tetraploid pattern unless these cells are numerous. Tetraploidy in benign urothelial cells can be explained by a transition status through the S or G2 phase of the cell cycle or by endoreplication as a response to cellular stress [60]. In contrast, unbalanced numerical changes of 1 or more chromosomes (e.g. 2, 3, 5) or loss of 9p21 is virtually specific for neoplasia in bladder cytology. Notably, pelvic irradiation (e.g. of prostate cancer) often leads to permanent chromosomal aberrations, emphasizing the need of appropriate clinical information and consideration of the typical post-irradiation cytomorphological changes. Despite the radiation-induced chromosomal aberrations, the risk of bladder cancer after irradiation for prostate cancer is only marginally increased [61]. In contrast to unbalanced polysomies, we have never observed deletions of 9p21 in post-irradiation specimens from patients without a history of UC cancer. Thus, a positive FISH result in a patient with a history of pelvic irradiation (e.g. of prostate cancer) does not prove cancer unless there is unequivocal 9p21 deletion.

Controversies and Criticism

There are ongoing controversies and opinions against a wide application of UroVysion in routine practice [8, 62, 63]. Some experts emphasize the high accuracy of cytology in detecting high-grade lesions as compared to the minor benefit gained from better diagnosis of clinically harmless low-grade lesions by FISH. There are also concerns about the cost efficiency of the relatively expensive UroVysion testing. The issue of the low-positive predictive value of FISH if applied in unselected patients with hematuria is also stressed [8, 33, 62]. Renshaw [63] questions the reproducibility in actual clinical practice. He suggests educational programs focusing on borderline or difficult urine cytology and UroVysion samples to provide a basis for evidence-based decisions concerning the best use of these tests in the real-life setting. Taken together, there are valid arguments speaking against a wide and unselected application of UroVysion testing including the comparatively low-positive predictive value in some situations, the limited clinical value of enhanced detection of low-grade UC at a high price, and general concerns about cost efficiency. Nevertheless, the majority of studies and our daily experience suggest that UroVysion FISH can be an excellent diagnostic tool to overcome the limitations of cytology. However, FISH needs to be used wisely in well-defined situations.

Conclusion

The existing data and our experience suggest that multiprobe FISH is a helpful tool for improved diagnosis in difficult fields of urinary cytology. However, FISH analysis should be performed by persons with experience in cytopathology and not be delegated to purely technical staff. The FISH results must be interpreted in consideration of the clinical findings. Finally, UroVysion FISH is most rewarding in atypical cytological findings but redundant in clearly positive high-grade cytology.

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