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INTERPHASE FLUORESCENCE IN-SITU HYBRIDIZATION IN THE DIAGNOSIS OF BLADDER CANCER

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1. ABSTRACT

Interphase FISH is a technique that uses fluorescent molecules to detect chromosomes or specific regions of DNA. It is a rapid and powerful technique for detection of cytogenetic abnormalities in malignant cells independent of their cell cycle status. Using variety of pericentromeric and locus-specific probes, numerical chromosomal changes (aneusomy) as well as loss or gain/amplification of specific genetic regions can be detected in clinical samples. Numerous studies have identified genetic alterations at the DNA level, occurring in the pathogenesis of variety of human neoplasms including bladder cancer, some of which can be used for detection, prognosis, and as intermediate endpoints for evaluating the response to therapy.

Recently, sensitivity and specificity of a multicolor FISH assay consisting of four probes (3, 7, 17 and 9p21) was analyzed in several prospective and retrospective studies. The data suggest that this method applicable to voided urine specimens may allow safe extension of the interval between cystoscopies in routine surveillance of patients with transitional cell carcinoma of the bladder. FISH analysis of cells isolated from bladder washings or voided urine is also holding promise for monitoring of treatment outcome and predicting recurrence and progression of the disease. Therefore, this technique can be an important aid in the efforts to reduce mortality from transitional cell carcinoma of the bladder, since it increases our ability to prevent progression to incurable muscle invasive disease.

2. FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

Interphase FISH is a technique that uses fluorescent molecules to detect chromosomes or chromosomal regions. It is a rapid and powerful technique for detection of cytogenetic abnormalities in malignant cells independent of their cell cycle status. FISH involves preparation of short sequences of single-stranded DNA probes, which are complementary to the DNA sequences subject to analysis. These probes then hybridize to the

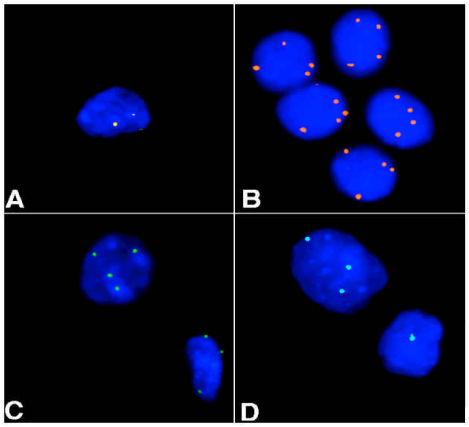

Figure 1. Multicolor fluorescence *in situ* hybridization performed on a destained ThinPrep slide prepared from a voided urine specimen from a patient with bladder cancer. Using the commercially available multitarget assay (UroVysion, Vysis, Downer's Grove, IL), the cells show two 9p21 signals (A-gold), however, they display aneusomy of chromosome 3 (B-red), 7 (C-green) and 17 (D-aqua).

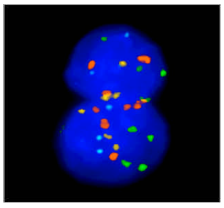

Figure 2. A composite UroVysion image of two malignant transitional cells from case in Figure 1 (9p21 band (gold), chromosome 3 (red), 7 (green) and 17 (aqua), counterstained with DAPI).

complementary DNA and because they carry a fluorescent label, identification of the presence or absence of the studied sequence, chromosome, or chromosomal region is possible. Through a fluorescence microscope, the probe signals appear as compact fluorescent spots where the probe has hybridized to the target DNA. Using variety of pericentromeric and locus-specific probes, numerical chromosomal changes (aneusomy) as well as loss or gain/amplification of specific regions of DNA can be detected in clinical samples. Using multi-color FISH, multiple targets can be analyzed simultaneously in a single hybridization reaction performed on a single glass slide.

Interphase FISH is increasingly utilized as an adjunct method in the diagnosis of both hematologic malignancies and solid tumors (1). Recent studies have identified genetic alterations at the DNA level - including gain or loss of genes, chromosomal segments, and chromosomes, occurring in the pathogenesis of variety of human neoplasms including transitional cell carcinoma of the bladder (bladder cancer), some of which can be used for detection, prognosis, and as intermediate endpoints for evaluating the response to therapy (2-4).

3. GENETIC ALTERATIONS IN THE EVOLUTION OF BLADDER CANCER

Multiple anomalies appear to constitute part of the multistep carcinogenetic process by which clinically and pathologically recognizable bladder cancers develop (5). The earliest and possibly initiating molecular genetic event in papillary urothelial tumors is the loss of a portion of chromosome 9 (9p21 region containing the p16/CDKN2A gene). This alteration has been consistently reported by many investigators using a variety of different techniques including interphase FISH and assessment of loss of heterozygozity (LOH) (2-4,6-9). In tumors in which this is the only chromosomal change, there appears to be little tendency towards aggressive behavior (8). In contrast, changes in chromosomes 3, 11 and 17 have been associated with a strong likelihood of progression (8). Alterations of chromosome 17, which correlate with mutant p53 protein expression, have been observed in the carcinoma *in situ* form of noninvasive bladder cancer and in invasive bladder cancers in studies utilizing LOH analysis as well as immunohistochemistry (5,10-13). Other changes occurring later in the progression of bladder cancer include loss of chromosome 16 and 18, loss of 3p, 5q, 6q, 2q, increase in chromosome 7 and portions of chromosome 8, along with many reported translocations detectable by conventional cytogenetics or interphase FISH (8,10,13-15).

The hypothesis that distinct genotypic patterns are associated with early and later stages of bladder tumor development is supported by results of studies utilizing comparative genomic hybridization (16, 17). In general, pTa carcinomas are thought to represent genetically stable tumors that usually accumulate few cytogenetic changes. In contrast, invasive bladder tumors (pT1-pT4) are genetically unstable and often display a high number of cytogenetic alterations.

4. APPLICATION OF FISH TO CLINICAL SPECIMENS

The capability of FISH to detect bladder cancer cells in clinical specimens has been addressed in multiple studies. Using probes for chromosomes 7, 8, and 9, Marano *et al* reported a good concordance of hybridization efficiency between the bladder barbotage specimens and touch biopsy slides of the excised tumors (16). In respect to the type of cell preparation used for FISH, several options are available and individual preferences exist among different laboratories. In numerous studies, FISH performed well in cytologic material using direct smears, cytospins or sedimentation preparations fixed in Carnoy's fixative (Methanol/Acetic acid 20:1) (19-25). As an alternative, Cajulis *et al* evaluated the feasibility of FISH on previously stained direct smears (Papanicolau and Diff-Quick) and demonstrated very good results (26). In recent years, liquid-based cytology has emerged as an alternative to conventional cytopreparatory methods and in particular, the ThinPrep system has found broad acceptance in non-gynecologic cytopreparation (27). Using ThinPrep slides, Florentine *et al* showed good results when analyzing specimens from body cavity effusions by dual-color FISH with pericentromeric probes to chromosomes 3, 8, 10 and 12 (28). Similar results can be obtained when performing multicolor FISH on either fresh or previously stained slides prepared from urine cytology specimens by the ThinPrep technique (29) (Figure 1, 2).

One of the limitations of FISH in routine evaluation of urinary specimens may be the relatively limited availability of this technology and the lack of experience necessary for performing this assay among clinical laboratories. Also, the relatively high costs traditionally associated with this type of analysis may be a limiting factor. However, the substantially less complex cell conditioning and hybridization procedures introduced by the directly labeled probes in the recently developed multicolor assays will remarkably facilitate more widespread use of this technique. The costs of the reagents in such assays (in the range of $80-100 per test) are comparable to those of other ancillary tests that have been utilized in bladder cancer detection programs.

5. EFFICACY OF FISH IN BLADDER CANCER DETECTION IN COMPARISON TO URINARY CYTOLOGY

Urinary cytology has been traditionally used as an adjunct to cystoscopy in the detection of bladder cancer. However, the sensitivity of cytology is limited, especially in the detection of low-grade non-invasive tumors (stage pTa). Therefore, the efficacy of ancillary techniques used for bladder cancer detection (including FISH) is typically compared to the sensitivity and specificity of cytology. Junker *et al* showed in a study of 55 voided urine samples that FISH for chromosomes 7, 8, 9 and 12 was more sensitive than cytology (68.5% vs 50%) while demonstrating high specificity (30). In a study of 21 patients surveilled for tumor recurrence by Inoue and coworkers, the sensitivity of FISH (80%) was superior to that of cytology (40%) when detecting numerical aberrations of chromosomes 7 and 9 (31). Eleuteri *et al* found that using FISH and flow cytometry, detection of numerical aberrations of chromosome 9 and cellular DNA content, respectively, can complement information in cytodiagnosis and follow-up of patients with bladder cancer (32). In this study, DNA alterations were detected in 95% of 44 patients with biopsy proven carcinoma (32). Ishiwata *et al* performed FISH for chromosome 9 and 17 and compared its efficacy in detecting bladder cancer to urinary cytology and the bladder tumor antigen (BTA) test (33). In this study, the sensitivity and specificity of FISH was 85% and 95%, respectively. This compared to 32% and 64% sensitivity and 100% and 80% specificity of cytology and BTA test, respectively (33).

Genetic changes may be actually detectable even in the cystoscopically normal mucosa prior to the formation of tumor, as was recently demonstrated by Hartman *et al* (34) and Pan *et al* (35). Using FISH, numerical aberrations of chromosomes 7, 9, 10 and 11 were commonly found in both tumor and cystoscopically normal mucosa from the bladder of the same patients, demonstrating the linkage between the genetic abnormalities in the tumor and precursor changes in the cystoscopically normal mucosa. Thus, FISH offers the capability to not only detect the tumor sooner than cytology, but also to detect the precursor changes in the mucosa prior to macroscopic or microscopic tumor formation. This feature of FISH requires sensitive approach to the results of this test in terms of patient management. Patients with cystoscopically negative findings and/or negative bladder biopsy may require intensified follow-up with re-biopsy. As was recently demonstrated, the majority of such patients develop grossly and microscopically obvious tumors within a period of several months (24).

Most recently, a set of 9 probes (9, 7, 17, 3, 8, 11, 18, Y and 9p21) was comprehensively evaluated for their sensitivity and specificity in detection of neoplastic cells in the urine from patients with biopsy proven urothelial carcinoma (25). In conjunction with the findings of this extensive analysis, sensitivity and specificity of a multicolor FISH assay consisting of four most informative probes from this set (3, 7, 17 and 9p21; UroVysion, Vysis, Downers Grove, IL) was evaluated in prospectively obtained urine specimens by Halling and coworkers from the Mayo Clinic (24). This study demonstrated that using this combination of probes, FISH has higher sensitivity than cytology in detection of carcinoma when monitoring patients for tumor recurrence (81% vs. 58%) while maintaining high specificity of cytology (96%). The probes detected 20 of 21 cancer cases (95%). In addition to all cytology positive tumors, FISH detected 7 of 8 cancers that were negative (or equivocal) by standard urine cytology. This study represents an important step in the clinical validation of the studied probe set and defined its predictive potential in the clinical setting. In a follow-up study, this group of investigators demonstrated that the combined sensitivity of cystoscopy and FISH (97%) is superior to that of cystoscopy combined with urine cytology (89%) (36). Similar results were reported by Bubendorf *et al* who evaluated the UroVysion FISH assay in a prospective series of 97 voided urine and 68 bladder washing specimens (37). In their hands, FISH detected 73% of pTa tumors, 100% of pT1 and 100% of pT2-4 tumors. In addition, FISH was positive in five of 10 patients with negative follow-up cystoscopy results. Similarly to study of Halling *et al* (24), subsequent recurrence was found in four of these patients but never in patients with negative FISH result (37). Alternatively, when applied to destained slides targeting the cytologically equivocal cells (“atypical transitional cells”), the UroVysion assay enables to identify malignancy with high sensitivity (93%) and specificity (98%) as was demonstrated in a study of 45 voided, instrumented and bladder barbotage specimens (38). Recent multi-institutional study evaluated the sensitivity and specificity of this multicolor assay and confirmed that this set of probes has the highest combination of sensitivity and specificity when compared to other adjunctive tests such as urinary cytology and BTA*stat* test (39). Because the UroVysion FISH assay showed overall sensitivity comparable to cystoscopy but higher specificity, the data suggest that it may be the first test applicable to voided urine in order to allow safe extension of the interval between cystoscopies for routine bladder cancer surveillance (39).

6. PREDICTION OF TUMOR RECURRENCE AND PROGRESSION

Aside from the utility in tumor detection, results of several studies suggest that the chromosomal changes

detectable by FISH might predict recurrence in patients with primary non-invasive (pTa) or superficially invasive (pT1) transitional cell carcinoma. In a study of formalin-fixed paraffin-embedded tumors of 52 patients, Watters *et al* showed that aneusomy of chromosomes 7 and 17 predict recurrence in patients with pTA/pT1 cancers (40). The aberrations were found in 31% of patients with subsequent recurrent disease while no aneusomy was seen in patients without recurrence. Similar correlation between aneusomy of chromosomes 7 and 17 and tumor recurrence and progression was observed by Pycha *et al* in a series of 50 patients with transitional cell carcinoma, 29 of which had primarily non-invasive stage pTa tumor (41). Bartlett *et al* found aberrations of chromosomes 7, 9 and 17 in 68% of superficial carcinomas and demonstrated that the changes are predictive of recurrence independent of tumor polyploidy (42). Recently, Cianciulli *et al* reported that chromosome 7 and 17 aneusomy shows greater differences between pT1 and pT2-3 tumors than between stage pTa and pT1, confirming that chromosome 7 and 17 aneusomy could be predictive of adverse outcome in a subgroup of patients with superficial tumors at presentation (43).

7. MONITORING RESPONSE TO ADJUVANT THERAPY

Immediate intravesical immuno- and chemotherapy at the time of transurethral resection may be useful in eliminating or delaying the recurrence and progression of bladder cancer. Pycha *et al* analyzed whether cytogenetic abnormalities in bladder cancer patients are modified or eliminated by instillation therapy by Bacillus Calmette-Guerin (BCG) and Mitomycin C (44). Analyzing numerical aberrations of chromosomes 7, 9 and 17, they reported that the chromosomal patterns in patients treated with BCG either remained stable (53%) or progressed to a more aggressive pattern (47%). In the mitomycin C treated group, 40% of patients showed stable and 50% showed progressive chromosomal alterations. Only one patient in this group changed to a regular diploid chromosomal pattern.

The reactive changes induced by the adjuvant therapy commonly interfere with the ability of cytology and cystoscopy to accurately evaluate the response to therapy. Stewart *et al* analyzed the relative sensitivity of FISH (UroVysion) and urine cytology in patients receiving BCG therapy and demonstrated that FISH significantly improves the detection of bladder cancer recurrence in this setting (45).

8. CONCLUSION

Fluorescence *in situ* hybridization is a highly specific and sensitive tool with a great potential in the clinical management of bladder cancer. FISH analysis of cells isolated from bladder washings or voided urine is holding promise for early bladder cancer detection, monitoring of treatment outcome, and predicting recurrence and progression of the disease. The recent results show that compared to cytology, FISH allows earlier detection of bladder cancer recurrence and therefore, using this technique can be an important aid in the efforts to reduce mortality from urothelial carcinoma resulting from progression to potentially incurable muscle invasive disease. The recently introduced and clinically validated multicolor assay combining directly labeled probes to several highly informative targets will facilitate the implementation of this type of assay in routine urologic practice.

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