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Evaluation of DNA Flow Cytometry as a Screening Test for Bladder Cancer

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Abstract At this present time, we feel that there is no role for DNA flow cytometry (FCM), or indeed DNA studies by any other method, to be used as a screening procedure for patients with no prior history of bladder cancer due to the high false-positive rate found when monitoring exfoliated urothelial cells. On the other hand, for patients who have had a superficial transitional cell carcinoma (TCC), which has a documented 50% recurrence rate, and depending on pathological features, a progression rate from 7 to 45%, DNA FCM provides a sensitive method to predict future disease recurrence. It provides an extremely effective way to predict future progression and further acts as a method to monitor changes in the malignant potential of the patient's disease.

For those patients with a past history of superficial TCC who develop abnormal ploidy without any overt tumor, 80% will, within the next four years, suffer a disease recurrence. For the patient who has a Ta TCC and receives intravesical Bacillus Calmette-Guerin (BCG), the development of abnormal ploidy in bladder washing specimens is the single best indicator for future disease recurrence. Similarly, a negative DNA FCM of a bladder washing at six months after intravesical therapy is an excellent predictor of no further occurrence. In patients with superficial TCC, ploidy of the initial and recurrent tumor predicts for future progression. Half of those patients with stage Ta bladder cancer with two successive aneuploid bladder tumors develop muscle invasive disease within one year, while three-fourths develop advanced disease within two years after recurrence of their second aneuploid lesion.

There is a group of patients for whom false-negative results may present a serious problem. These are patients with microhematuria of unknown etiology. We will illustrate how a protein counterstain and two-parameter DNA-protein histograms may be used to eliminate the signals from the extraneous leukocytes and permit detection of relatively small numbers of aneuploid cells by FCM.

It is highly probable that bladder cancer, like other types of cancer, develops as a result of a series of somatic mutations. Two-parameter DNA fluorescent antibody study of normal, mutant and deleted oncogenes or tumor suppressor genes offers tremendous promise in monitoring the clinical course of bladder cancer. As a paradigm for the study of bladder cancer, we will illustrate FCM detection of the p53 protein product in archival prostate tissue.

Monoclonal antibody studies offer exciting prospects for the better detection and characterization of TCC. Screening for this disease may, in the future, employ a dipstick method. For people with a past history of bladder cancer or who are found to have current disease, the number of markers which can be measured by either FCM or image analysis will be the key to determining the malignant potential and the sensitivity of the tumor to therapy. © 1992 Wiley-Liss, Inc.

Key words: bladder cancer, DNA FCM, screening methodologies

DNA flow cytometry (FCM) is an unsuitable screening test for bladder cancer if screening is defined as testing a large population of people who have no history of a given disease but who, for various reasons, are felt to be at increased risk of developing that disease. One reason for this conclusion is the high false-positive rate found when monitoring exfoliated urothelial cells from patients who have no history of bladder cancer. For patients with benign prostatic hypertrophy or active renal stone disease, we found 11–14% false-positive DNA histograms [1]. This rose to 36% for a group of smokers. While it is reasonable to argue that these people are at increased risk of developing transitional cell carcinoma (TCC) in the future, it is not possible to know how great this risk is or how to follow these people since we have no proven cost-effective way to monitor them for the development of TCC. At this time, we feel there is no role in the screening of patients with no history of bladder cancer for DNA FCM or DNA

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studies by any other method [1]. In a recent publication the NCI Bladder Flow Cytometry Network made a similar recommendation with regard to screening [2].

On the other hand, patients with a history of Ta T1 TCC have a 50% recurrence rate and, depending on pathological features, the chance of disease progression varies from 7–45% [3]. In these patients, DNA FCM of bladder washing specimens provides a sensitive method for predicting future disease recurrence. Several groups have reported that using a combination of DNA FCM and cytology in this patient population gives a bladder cancer detection rate of 90–94%. Further, it provides an extremely effective way to predict future tumor progression and, as reflected by a change in ploidy during follow-up, acts as a method for monitoring changes in the malignant potential of the patients' disease [1,3–7].

Bladder washing samples should thus be used only for patients with a past history of TCC. Groups to be targeted are patients who have had a carcinoma *in situ* (CIS), Ta, or T1 tumor resected. Bladder washing is used to sample the rest of the bladder mucosa for field-change disease. Also, ploidy differences may be found between the overt tumor and the bladder washing sample. Bladder washings monitor patients during follow-up and after intravesical therapy. In patients who have received systemic chemotherapy for invasive disease, bladder washing samples can be used to monitor alterations in the mucosa, but not for recurrent muscular disease [7].

We have looked extensively at the use of both urine (BU) and bladder washings (BW) as a method of evaluating patients for recurrence of TCC. We have previously published our detection, false-positive, and false-negative rates, as well as the percent unsatisfactory samples obtained using both methods [1,4,8–10]. One of the major drawbacks of FCM has been the lack of a suitable shipping method to send samples to a reference laboratory. We have overcome this with a fixation method (methanol/acetic acid, 20:1 v:v) in which the fixative is poured directly into an equal volume of urine or washing fluid [8]. The method requires no refrigeration or centrifugation at the collection site and has the added advantage that the fixative decreases leukocytes and red blood cells, resulting in decreased debris in the histogram sample. Finally, the reference laboratory can use the same sample for both flow cytometry and cytology. It does not, however, eliminate the problem of squamous cells in the sample.

In order to further improve the histograms and make interpretation easier, we have investigated the use of a dual-parameter DNA/protein stain with propidium iodide (PI) and fluorescein isothiocyanate (FITC).

Since we obtain histograms only from patients with a past history of TCC, the importance of an improved histogram is in detecting minor changes during follow-up, thus predicting both recurrence and alterations in malignant potential. However, in the context of this paper, the question is whether this manipulation of the histogram will allow recommendation of DNA FCM as a screening test. We examined urine and/or bladder washing specimens from 73 patients with active TCC or a past history of this disease. At the time of examination, 22 patients had active disease and 51 had a past history. All patients were cystoscoped to confirm the disease status at the time the sample was obtained.

The advantage of the dual-parameter stain is its ability to eliminate the signals from extraneous leukocytes and squamous cells. The effect is to clarify histogram interpretation by confirming the absence of aneuploidy, confirming a suspicious aneuploid peak, or finding a previously undetectable aneuploid peak. Since we are evaluating this procedure as a screening test, we classified all suspicious histograms as either negative or positive since all non-negative histograms would have to be investigated.

Of the 22 patients with active TCC, 59% were positive by DNA-only FCM. After dual-parameter DNA/FITC analysis, 86% were positive. The three mis-calls were two grade I and one grade II tumors, all of which were superficial. Two of 11 urine samples and 1 of 11 bladder washing samples were missed. This appears to be a low detection rate; however, Huland and colleagues reported a detection rate of 57% by FCM alone for patients with grade III tumors [11].

DNA analysis of the 51 patients with a past history of TCC showed that 43 (84%) were negative and 8 (16%) were positive. Based on the active disease group, we expected that dual-

parameter analysis would increase the "false"-positive rate; in fact, 41 (80%) samples were still negative and only 10 (20%) were positive after the DNA/FITC analysis. If all 73 patients are considered (as one would do in a screening setting), then DNA FCM alone would have missed 9 active tumors while dual-parameter analysis would have missed only 3. However, 11% of histograms that were called positive by DNA FCM were increased to 14% by dual-parameter analysis. These patients had no evidence of active disease. Thus, while there is considerable advantage to the use of the dual-parameter method in patients with a past history of TCC, we do not feel it is a suitable method for screening patients for TCC.

We have published extensively on the use of voided urine for DNA FCM [1,8,9]. If FCM is employed as a screening test, voided urine samples should be used. While it is reasonable to use washings in patients with a past history of TCC, it would be totally unacceptable in the screening setting. Bladder washings frequently provide better DNA histograms than voided urine; however, it has been shown that if the washings are obtained by inexperienced medical laboratory personnel, a large number (approximately 20%) of these histograms will be unsatisfactory due to low cell numbers [2]. Urine samples avoid this problem and patients need not be instrumented. Regardless of this, the weight of evidence is that urine does not give the same satisfactory results as bladder washings [12–14].

FOLLOWING PATIENTS WITH A PAST HISTORY OF TCC

For patients with a past history of TCC, the appearance of a positive DNA FCM during follow-up is evidence that the disease has or will recur. In our series, tumors recurred in 39% of such patients. Benson *et al.* (in press) report an 80% disease recurrence within four years.

For patients with Ta TCC who have received intravesical Bacillus Calmette-Guerin (BCG), the Memorial Sloan-Kettering group has shown that development of abnormal ploidy in a bladder washing specimen obtained 6 months after therapy is the single best indication of future disease activity. The mean estimated time to progression for 65 patients was 38 months. If the FCM was positive at 6 months, the mean time to progression was 30 months, and if the FCM was negative at 6 months, the probability of progression-free survival at 30 months was 85% [7].

While we have not published on such work, we have made similar observations. The presence of a positive histogram is not only an indication of disease recurrence, but a histogram that changes from diploid after intravesical therapy to aneuploid during follow-up indicates an increase in the malignant potential of the patient's disease as well. It is our opinion that washings submitted to DNA FCM in this setting are an extremely useful clinical tool.

PATIENTS WITH CIS, Ta, OR T1 TUMORS

For patients with a history of Ta T1 TCC, we know that there is a 50% recurrence rate. Depending on the grade and stage, the chance of disease progression varies from 7–45% [3]. It can be calculated that approximately 1% of all patients who presented with Ta disease and 10–15% who presented with T1 disease will die of TCC this year. The challenge is to identify this small subset of patients who need more aggressive therapy.

The ploidy of the initial and recurrent tumors from patients with superficial TCC predicts future progression. In a study of stage Ta bladder cancers, we, with John Fitzpatrick [3], observed that half the patients who had two successive aneuploid bladder tumors developed muscle-invasive disease within one year while three-fourths developed advanced disease within two years. It must be remembered that the progression rate was only 9% in the 200 patients overall. It is our opinion that the ploidy of Ta T1 disease is very helpful in predicting which patients are likely to progress. While aneuploidy predicts for aggressive disease, we feel that diploid Ta T1 tumors pose little risk to the patient. Tribukait [15] has published very similar conclusions. In Carateros' paper [16], aneuploidy was found in 8% of Pa, 46% of P1, 73% of P2, and 87% of P3 tumors. Of 47 cystectomies for TCC reported by Shaaban *et al.* [17], only 3 were diploid and none of these had node-positive disease.

Ploidy is not the only histogram feature to be considered. The importance of estimated S-phase activity and the percent of hyperdiploid

cells have also been evaluated as markers of altered malignant potential.

In patients undergoing cystectomy, Shaaban [17] examined the percent of S-phase activity in relation to positive nodes. If the percent of S-phase activity was <10, positive nodes occurred in 33%; if 10–20, 50%; >20, 100%. The problem is the relatively small numbers in each group.

Tachibana *et al.* [18] have recently published on the importance of the percent of hypertetraploid cells in DNA histograms. Patients whose histograms showed >5% of cells with DNA index of 2.2 were considered to be hyper-5C or cells exceeding 5C. Again, this study mixed grade and stage so that when subgroups were determined, the numbers in each group were relatively small. This notwithstanding, the three-year and five-year survival rate was 92% and 89% for 149 patients with low (≤5%) hypertetraploid cells, while for patients with ≥5% of cells in the hypertetraploid region, the three- and five-year survival figures fell to 33% and 10%, respectively [18]. Interestingly, the percent of hypertetraploid cells in S-phase did not add to the model. It is useful to compare patients in Tachibana's series to patients estimated to be hyper-5C by Quantitative Fluorescence Image Analysis (QFIA). Hemstreet *et al.* [19] reported that 70/88 low-grade tumors were diploid, over 21% of the patients died of TCC, and that the single most important predictors of recurrence and death were aneuploidy and the overall percent of hyper-5C cells [19].

At this time, histograms need to be analyzed for ploidy, percent of S-phase, and percent of hyper-5C cells. It can be asked, however, whether all superficial tumors should be submitted to histogram analysis if only 7% of Ta tumors and 45% of T1 tumors progress [3]. Practically speaking, the answer is probably no. Grade I tumors are mostly diploid while the majority of Grade III tumors are aneuploid, so additional information would be obtained in a much more cost-effective method if DNA analysis was performed on Grade II lesions [16,20,21].

It has always been our opinion that a bladder washing or urine sample in patients with a history of TCC should be submitted to both DNA FCM and cytology to give the best possible chance of disease detection. Nothing has occurred over the past several years to change this opinion. Further, knowing the ploidy of both the initial tumor and, more importantly, the recurrent Ta T1 lesions, gives important clinical information that is most valuable in Grade II tumors. Whether these tests should be carried out by FCM or image analysis is probably irrelevant. Ploidy analysis by either test, in most cases, will give similar clinical information. The ongoing Southwest Oncology Group (SWOG) study comparing both methods will hopefully give a more definitive answer to this question. In the clinical setting, the most convenient test should be employed.

FUTURE CONSIDERATIONS

It is highly probable that bladder cancer, like other types of cancer, develops as a result of a series of somatic mutations. Two-parameter DNA fluorescent antibody studies of normal, mutant, and whole or partial deletions in oncogenes or tumor suppressor genes offer tremendous promise in monitoring the clinical course of bladder cancer. A likely candidate for study is the p53 gene, which in its normal state is a tumor suppressor gene, but abrogates its function after mutation or deletion. Morkve *et al.* [22] have evaluated TCC by simultaneous DNA and p53 expression FCM analyses. The study showed that simultaneous analyses could utilize either fresh or paraffin-embedded tissue using the p53 monoclonal antibody, 1801. When the histogram was analyzed for the p53-positive and p53-negative cells, the p53-positive histogram was enriched for S and G2 cells. It is our opinion that over the next few years, such dual-parameter studies with p53 and other genes yet to be described will be routine in determining the malignant potential and response to therapy in patients with bladder cancer. Indeed, the monoclonal studies of Drs. Fradet and Hemstreet (in this volume) offer exciting prospects for better characterization and detection of TCC.

In the near future, we expect that there will be a screening study for TCC that uses voided urine submitted to some variation of the dipstick methods presently being investigated. It is highly unlikely that either FCM or image-based methods will ever be cost-effective in a screening setting. However, for patients with a past history of bladder cancer, for those with recur-

rent disease, and for those being followed after therapy, we will come to rely on a number of markers that can be measured by FCM or image analysis as the key to determining the malignant potential of tumors and, hopefully, their sensitivity to treatment.

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