Is Aneusomy of Chromosome 9 Alone a Valid Biomarker for Urinary Bladder Cancer Screening?

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Abstract. *Background: Detection of genetically-changed tumor cells in the urine is one of the new approaches for the screening of bladder carcinomas. In a previous study, numerical aberrations of chromosome 9 were found in 85.18% of bladder tumors studied by the fluorescence in situ hybridization (FISH) technique. The purpose of the present study was to investigate whether chromosome 9 aneusomy alone is a valid, cost effective, biomarker for bladder cancer screening. Materials and Methods: Twenty-seven voided urine specimens obtained from 22 bladder cancer patients, either at initial diagnosis or at the follow-up, were analyzed by the FISH technique with the centromeric probe specific for chromosome 9. Results: In all except 2 out of the 13 specimens with a histological confirmation of cancer, FISH analysis showed aneusomy 9 (sensitivity 84.61%). Among 6 cases with a negative cystoscopy but a positive FISH analysis, 3 recurred within the following 2 months, while 2 no-recurrent patients continued to show positive FISH findings after 6 months. One patient was considered to be false-positive. Four cases with a negative cystoscopy showed disomy 9 and 2 of them recurred. Conclusion: Aneusomy 9 has a high sensitivity (84.61%) for the detection of bladder cancer. Patients with a negative cystoscopy but with aneusomy 9 should be kept under close clinical surveillance for potential disease recurrence. However, negative FISH results might not be a negative predictor for disease recurrence. Our results encourage further studies with a large number of patients and a long-term follow-up with concurrent FISH analysis.*

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Bladder cancer is a genetically heterogeneous disease, with multiple genetic changes being implicated in its development. However, no specific abnormality associated with bladder cancer has yet been established. Conventional cytogenetics and molecular genetic techniques have revealed non-random changes in bladder cancer, while certain genetic imbalances have been found to be correlated with tumor grade and/or stage. The fluorescence *in situ* hybridization (FISH) technique using the centromeric specific DNA probes is a valuable tool for the rapid detection of numerical chromosomal aberrations of malignant cells (1-10).

Following tumor resection, recurrence is found in a large percentage of bladder cancer patients, while progression to invasive disease accounts for 10-20% of recurrent cases. Therefore, the early detection of tumor recurrence is of major clinical importance and requires close surveillance for appropriate treatment strategies. The follow-up of patients is based on cystoscopy. From a clinical point of view, it is important to develop highly sensitive, non-invasive and comfortable methods for the follow-up of these patients. The detection of genetically-changed tumor cells in the urine is one of the new approaches for the screening of bladder carcinomas. A multicolor multitarget FISH assay using a panel of probes has recently been shown to have high sensitivity and specificity for detecting bladder carcinoma (11-18). In our previous study, (8) numerical aberrations of chromosome 9 (loss or/and polysomies) were found in 85.18% of bladder tumors studied by the FISH technique. Taking into account the cost of the multitarget FISH assay, whether numerical aberrations of chromosome 9 alone constitute a potential, cost-effective, biomarker for bladder cancer screening was investigated.

Materials and Methods

Twenty-two patients,17 males and 5 females, were included in this study and a total of 27 samples of urine were examined with the FISH technique. All the patients underwent cystoscopic examination, either for a transurethral resection (TUR) of the

Table I. *Histopathological characteristics and urine FISH analysis of 22 bladder cancer patients.*

	Initial diagnosis		Follow-up		
Patient/ Gender/Age	Histopathological findings (Stage/Grade)	FISH analysis	Cystoscopy	Histopathological findings (Stage/Grade)	FISH analysis
1-M-67	pT1/II	Positive			-
2-M-49	pT1/IIa	Positive			-
3-M-65	pT1/IIa-IIb	Negative			-
4-M-80	pT1a/IIa	Positive			-
5-M-80	pT1b/IIb-III	-	Positive	pT2/III	Positive
6-M-74	pT1b/IIa	Positive			-
7-M-65	pT1b/IIb	Positive			-
8-M-70	pT2/III	Positive			-
9-M-58	pT1b/IIb	Positive			-
10-M-60	pT1a/IIa	Positive			-
11-M-62	pT1/IIa	Positive			-
12-F-51	pT1/IIa	-	Positive	Follicular cystitis	Negative
13-F-53	pTa/I	-	Positive	Chronic cystitis	Positive
13			Negative		Positive
14/M/62	pT1b/III	-	Negative		Positive
14			Positive	pT1b/III	-
15-F-85	pT1a/I-IIa	-	Negative		Positive
15			Positive	pT1a/IIa	Positive
16-F-70	pT1b/IIa-IIb	-	Negative		Negative
17-M-79	pT1a/IIa-IIb	Negative			
17			Negative		Negative
18-F-73	pT2/III	-	Negative		Negative
19-M-70	pT2/III	-	Negative		Positive
19			Negative		Positive
20-M-79	pT1b/II	-	Negative		Positive
20		-	Negative		Negative
21-M-67	pT1b/IIa-IIb	-	Negative		Positive
21			Positive	pT1b/IIa-IIb	-
22-M-61	pT1a/III	-	Negative		Negative

bladder tumor at initial diagnosis (11 patients) or for the follow-up, after a previously diagnosed bladder carcinoma (12 patients). The latter patients presented either recurrence of the disease or had no visible tumor at cystoscopy. Before the cystoscopy, voided urine specimens had been collected for FISH analysis and centrifuged at 1000 rpm for 8-10 min. The pellet was treated with a hypotonic solution of 0.075 M KCl for 30 min, followed by 3 fixations in 3:1 methanol/acetic acid and were then refrigerated until the FISH study was performed. The FISH technique was applied on recently made slides of the methanol/acetic acid fixed cells using DNA probe D9Z3 specific for chromosome 9 (chromosomal region 9q12) (Cytocell Technologies, Cambridge, UK), according to the manufacturer's instructions. The hybridization of the probe with the cellular DNA site was visualized by a fluorescence microscope NIKON E600 with a triple filter DAPI / FITC / TEXAS RED. Positive chromosome signals appeared as red or green spots in the nuclei. A minimum of 250 cells were evaluated for each case. A case was counted as aberrant if more than 10% of the cell nuclei showed loss or/and gain of signals for chromosome 9.

Results

Fifteen patients had a cystoscopic examination positive for malignant disease, either at initial diagnosis or at the follow-up. Among them, a histological confirmation of transitional cell carcinoma (TCC) was made in 13 cases, while in 2 cases follicular cystitis and chronic cystitis were found, respectively. The results of the FISH analysis, as well as the histopathological characteristics of the bladder tumors, are provided in Table I. A representative example of FISH analysis is shown in Figure 1. In all cases except 2 with a histological confirmation of TCC, the FISH technique showed aneusomy of chromosome 9 (monosomy or/and polysomies) (sensitivity 84.61%). In the case with a histological confirmation of follicular cystitis, the FISH technique showed disomy 9, while in the case with chronic cystitis FISH analysis revealed aneusomy 9. Although, the latter case did not recur

after 6 months, a new sample of the urine continued to show aneusomy 9. Interestingly, a positive cytological examination of the urine was subsequently revealed. Nine patients at the follow-up had a cystoscopic examination negative for malignant disease. Among them, the FISH technique showed disomy 9 in 4 cases (cases 16, 17, 18 and 22). Cases 18 and 22 recurred 6 and 2 months, respectively, after the FISH analysis. Notably, both cases had aneusomy 9 in about 10% of the cell nuclei. In the remaining 5 cases (cases 14, 15, 19, 20 and 21) FISH showed aneusomy 9. Three of the latter cases (cases 14, 15 and 21) had undergone TUR 1, 2 and 6 months, respectively, before the FISH analysis. All these patients recurred within the following 2 months. The fourth case (case 19) did not show recurrence 6 months after the first FISH analysis, while a second FISH analysis at that time continued to be positive. Finally, the fifth patient (case 20), had negative cystoscopic findings 2 months after TUR, while FISH analysis was positive. Six months later, recurrence had not occurred and a new FISH analysis did not show aneusomy 9.

Discussion

Bladder cancer has a high potential for recurrence and progression to more invasive stages. Cystoscopy is a basic method for diagnostic evaluation of patients with symptoms of bladder cancer as well as for the follow-up of bladder cancer patients in detecting disease recurrence. The cytological examination of the urine is an additional diagnostic tool to reveal patients for cystoscopic evaluation. However, the sensitivity of standard cytology in urinary samples is limited and, thus, unable to serve as a basis for therapy decisions. The development of highly sensitive, non-invasive and comfortable methods for diagnosis, prognosis and follow-up of bladder cancer patients is of major importance (12, 15, 17).

Bladder cancer is a genetically heterogeneous disease with multiple genetic alterations being involved in the development or the progression of this disease. The detection of genetically-changed cancer cells in the urine is critical for the diagnosis or characterization of biological behavior of bladder cancer. The FISH technique is a simple, rapid and powerful tool for detecting chromosomal aberrations on interphase nuclei using chromosome specific DNA probes (11, 15, 17, 19-21). Numerical aberrations of chromosome 9 are a common finding in bladder cancer and mainly include monosomy or partial deletions of the chromosomal material. They have been reported in early as well as in advanced disease (1, 7-8). Bartlett *et al.* (22) proposed that the loss of chromosome 9 from primary bladder tumors identified patients at high risk of recurrence and possible progression. In our previous study, we found numerical aberrations of chromosome 9 (monosomy or/and polysomies) in 85.18% of the bladder tumors studied by the FISH technique (8). In several studies, FISH was applied to urine samples using a centromeric probe specific for chromosome 9, but in combination with other DNA target probes (15, 16-17, 23-25). A multicolor multitarget (UroVysion, Vysis, Doweners Grove, Illinois, USA) FISH assay has recently been shown to have high sensitivity and specificity for detecting cancerous cells in the urine, also predicting disease recurrence (12-14, 26).

Taking into account the cost of the multiprobe FISH assay, we investigated whether aneusomy of chromosome 9 (monosomy or/and polysomies) alone represents a potential, cost-effective, biomarker for bladder cancer screening. In the present study, 27 voided urine samples from 22 bladder cancer patients were examined with FISH, using the centromeric probe specific for chromosome 9. In all except 2 out of the 13 samples obtained from patients with a histological confirmation of TCC, FISH analysis showed aneusomy 9 (sensitivity 84.61%). Moreover, in 10 patients with a negative cystoscopic examination, FISH analysis revealed disomy 9 in 4 cases, while in 6 specimens aneusomy 9 was found. Two of the patients with disomy 9 recurred after 2 and 6 months, respectively. Although, in both cases aneusomy 9 was found in about 10% of the cell nuclei, they were considered as FISH-negative, suggesting that negative FISH results might not serve as a negative predictor for tumor recurrence. Three of the cases with positive FISH findings recurred within the 2 months following the FISH analysis, supporting the hypothesis that aneusomy 9 might be a predictor for disease recurrence. However, 2 patients had not recurred at the 6-month follow-up, while they continued to present aneusomy 9. Although, these cases might be considered as FISH false-positive, they might have either a clinically undetectable TCC or a high probability of forming tumors over the time. Interestingly, in one of these cases a positive cytological examination was subsequently detected. Ishiwata *et al.* (15) suggested that urothelium with a normal appearance in bladder cancer patients may already have undergone chromosomal alterations leading to the subsequent development of visible cancer. Finally, 1 patient, who underwent TUR 2 months before the FISH analysis, had not recurred at the 6-month follow-up, while a new FISH analysis at that time did not detect aneusomy 9. This patient might be considered as FISH false-positive.

Tsukamoto *et al.* (23) used centromeric probes specific for chromosomes 9 and 17 on archival urine cytology specimens from patients with bladder TCC and suggested that monosomy 9 might be a prognostic marker for early tumor recurrence in patients with negative or equivocal cytology specimens. Jung *et al.* (24) applied FISH analysis to bladder irrigation specimens with the centromeric probe specific for chromosome 9 and the probe specific for the 9p21 region and showed that chromosome 9 monosomy was predictive of bladder tumor recurrence. In addition,

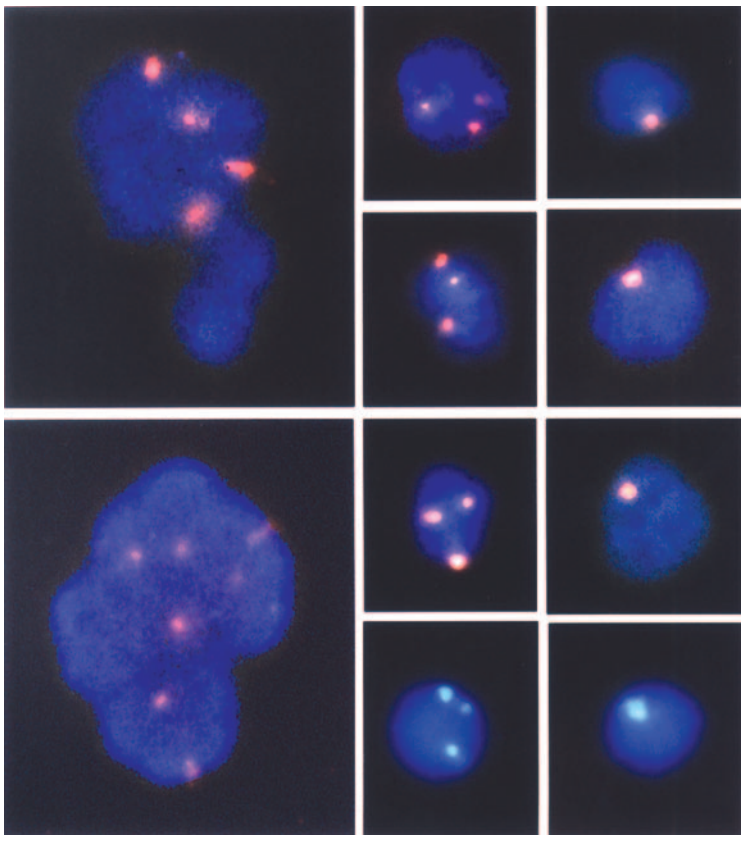

Figure 1. *Copy number of chromosome 9 (red or green spots) detected by FISH in the nuclei of tumor cells found in the urine of bladder cancer patients. Note the presence of aneusomy 9.*

Ishiwata *et al.* (15) used FISH analysis on voided urine specimens with the centromeric probes specific for chromosomes 9 and 17 and suggested that FISH analysis is characterized by a high sensitivity and specificity for bladder cancer. In addition, other studies using the multicolor FISH assay showed that positive multiprobe FISH analysis has a high sensitivity and specificity for detecting cancerous cells in the urine and also predicting disease recurrence (12-14, 25). Halling *et al.* (26) identified 11 false-positive cases by FISH, of which 7 had recurrent bladder carcinoma within 3 to 12 months following the positive FISH results. In addition, Bubendorf *et al.* (14) reported that a positive FISH result was highly predictive of tumor recurrence. Four out of the 5 patients with positive FISH results recurred at a mean of 7.9 months in their study, while none of the patients with negative FISH had a recurrence. Similarly, in a study by Scacel *et al.* (12), patients with positive FISH had tumor recurrence within 12 months. The authors reported that the FISH assay might be used for screening for bladder cancer, particularly in patients at high risk for occult neoplasia.

Although, the number of cases included in the present study was small and there was not a systematically synchronous FISH analysis at the follow-up of the patients, the results supported that aneusomy 9 has a high sensitivity (84.61%) for the detection of bladder cancer. Thus, aneusomy 9 might serve as a cost-effective biomarker for bladder cancer screening. For patients with a negative cystoscopy but with aneusomy 9, close clinical surveillance is recommended for potential disease recurrence. Negative FISH findings at the follow-up of patients might not be a negative predictor for disease recurrence. Our results encourage further studies with a large number of cases and a long-term follow-up of patients with concurrent urine FISH analysis.

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