Urol Res (2000) 28:57–61

ORIGINAL PAPER

T. Inoue · Y. Nasu · T. Tsushima · Y. Miyaji
T. Murakami · H. Kumon

Chromosomal numerical aberrations of exfoliated cells in the urine detected by fluorescence in situ hybridization: clinical implication for the detection of bladder cancer

Received: 24 June 1998 / Accepted: 1 July 1999

Abstract Numerical aberrations of chromosomes 7 and 9 of exfoliated cells from the urinary tract were examined using fluorescence in situ hybridization (FISH). To minimize contamination with inflammatory, squamous and normal transitional cells all samples were stained using Giemsa's solution and clusters of transitional cells were selected for FISH analysis. Samples collected from 21 patients who had cystoscopic monitoring for bladder cancer were used in this study. Cystoscopy was positive in seven patients and five biopsy-proven transitional cell carcinomas were identified. Among the patients with cancer recurrence, numerical chromosomal aberrations were detected in four cases (sensitivity: 80%) and positive cytology in two cases. This preliminary pilot study shows FISH can be a useful tool for the detection of recurrence during a follow up of bladder cancer.

Key words Chromosomal numerical aberration · FISH · Exfoliated cell · Bladder cancer

Introduction

Urine cytology has been used to detect urothelial tumors for diagnosis and follow up, but it requires experience and it is often difficult to identify low grade transitional cell carcinomas (TCCs) of the bladder because of a lack of significant cytomorphologic atypia [5]. Other methods for detecting low grade TCCs of bladder include cytogenetic studies and DNA measurement by flow cytometry. However, a cytogenetic study often requires a cell culture in order to determine a chromosomal aberration, and cell culture is time consuming to produce and almost impossible to achieve in the case of exfoliated cells. Furthermore, the study of the DNA content of exfoliated cells from the urinary tract using flow cytometry (FCM) has been reported to be less sensitive and specific than a conventional cytologic study [1]. The numerical aberration of chromosome 7 and 9 is most frequently found in transitional cell carcinoma of the bladder and early events in the evolution of bladder cancer [3, 6]. In our previous study, we indicated that the copy number of chromosome 7 was an indicator of ploidy, and the copy number of chromosome 9 was an indicator of deletion [4]. In this pilot study, numerical aberrations of chromosome 7 and 9 of exfoliated cells from the urinary tract were examined using the fluorescence in situ hybridization (FISH) technique. As a preliminary report, we discuss the potential for the clinical application of FISH in the early detection of bladder cancer in comparison with routine urinary cytology and cystoscopic examination.

T. Inoue · Y. Nasu · T. Tsushima (✉)
Y. Miyaji · T. Murakami · H. Kumon
Department of Urology,
Okayama University Medical School,
2–5-1 Shikata-cho, Okayama 700–8558, Japan
e-mail: tsushima@med.okayama-u.ac.jp
Tel.: +81 86 235 7285; Fax: +81 86 231 3986

Materials and methods

Twenty-one patients (aged 33–85, mean 67; one female) were enrolled in this study. They underwent cystoscopic monitoring for recurrence of superficial bladder cancer after surgery (trans-urethral resection, TUR) at the Okayama University Hospital from December 1995 to January 1996. Patient information is shown in Table 1. All patients had TUR as a previous definitive treatment.

Exfoliated cells were obtained from bladder washings at cystoscopy. Using a 19 or 21 French rigid cystoscope sheath, the bladder was filled with 300 ml sterilized phosphate buffered saline (PBS) containing 2.5% glucose (pH 7.2, 430 mOsm) and the entire voided specimen was centrifuged (2000 rpm for 2 min, twice). Sediment was smeared on silanized glass slides (DAKO, Kyoto, Japan) and quickly air-dried. Smears were fixed with 100% ethanol for 2 min. Diluted Giemsa's solution was applied to the slides for 5 min. If it stained weakly, the slide was restained for another 5 min. If the staining was too intense, the slide was soaked in 100% ethanol and excessive stain was removed. Using a light microscope, clusters of transitional cells were marked and microphotographs were taken.

FISH was performed on the Giemsa-stained smear preparations. Glass slides were immersed in 70% formamide, for 2 s and saline sodium citrate (SSC: 0.3 *M* NaCl and 0.03 sodium citrate) at pH 7.0 at 75°C for 2 min to denature double-stranded DNA. Satellite DNA probes specific for the pericentromeric regions of chromosomes 7 and 9 (D7Z1, D9Z5 digoxigenin labeled, Oncor, Gaithersburg,) were used. The probes were denatured at 72°C for 5 min and hybridization was performed at 37°C for 3 h or up to overnight. As a post-hybridization wash, the slides were immersed in 0.25× SSC at 70°C for 5 min. For fluorescent detection of hybrids and for counterstaining of nuclei, anti-digoxigenin-FITC fab fragments (Boehringer Mannheim, Germany) and propidium iodide (PI) were used, respectively. Evaluation was done by counting the number of signals in about 100 nuclei in marked clusters using a confocal laser microscope (LSM BG 200, Olympus). Two researchers performed the counting in an independent and blinded fashion and based on the criteria described by Waldman [12]. The FISH score was regarded as normal when the number of copies is two, for both chromosome 7 and 9. All other numerical combinations were regarded as indicators of positive numerical chromosomal aberrations.

Routine urinary cytologic examination was performed at a central laboratory using voided urine. This was obtained before cystoscopy. Class 5 was defined as being cytologically positive.

Table 1 Background of the patients (n = 21) (*TCC* transitional cell carcinoma, *AC* adenocarcinoma)

Age	
Range	33–85
Mean	66.7
Sex	
Male	20
Female	1
Previous histology	
TCC	20
AC	1
Previous grade	
G1	6
G2	6
G3	9
Follow-up duration (months)	
Range	4–63
Mean	18.3
Cytology	
Class 1	6
Class 2	9
Class 3	4
Class 4	0
Class 5	2
Bladder tumor on cystoscopy	
Yes	7
No	14

Results

Giemsa-stained preparations from bladder washings contained enough cells for FISH analysis. Transitional cells were observed among squamous cells and leukocytes. During the FISH procedure, cells rarely washed off the slide surface. FISH signals were clearly observed in the counter-stained nuclei (Figs. 1–3).

A summary of our results is shown in Table 2. Positive cytology (class 5) was observed in two cases (cases 1, 2). Positive cystoscopy was observed in seven patients (cases 1, 2, 4, 5, 6, 10, 20) and five biopsy-proven transitional cell carcinoma (TCC) of bladder were identified. Histology of the other two cases of positive cystoscopy were both without evidence of malignancy and showed follicular cystitis and papilloma respectively. Among the five cases with biopsy proven recurrence of bladder cancer, numerical chromosomal aberrations were detected in four cases. There were two cases (cases 1, 2) of grade 3 TCCs of the bladder. Both of these were strongly suspected to be malignant at the time of Giemsa staining. The other three cases (cases 4, 5, 6) of TCCs of bladder were all grade 1 and cytologically appeared as almost normal transitional cells (class 3), but FISH revealed numerical chromosome aberrations

Table 2 Summary of cytogenetics of exfoliated cells (*M* male, *F* female, *TCC* transitional cell carcinoma)

Case	Sex	Cytology	Tumor (cystoscope)	Histology	Copy no. chr. 7	Copy no. chr. 9	Chromosomal aberration
1	M	5	+	TCC, G3, T3a	2	3	+
2	M	5	+	TCC, G3, Tis	6	6	+
3	M	3	–		2	2	–
4	M	3	+	TCC, G1, Ta	2	2	–
5	M	3	+	TCC, G1, Ta	4	4	+
6	M	3	+	TCC, G1, Ta	2	1	+
7	M	2	–		2	2	–
8	M	2	–		2	2	–
9	M	2	–		2	2	–
10	M	2	+	Follicular cystitis	2	2	–
11	M	2	–		2	2	–
12	M	2	–		2	2	–
13	M	2	–		2	2	–
14	M	2	–		2	2	–
15	M	2	–		2	2	–
16	F	1	–		2	2	–
17	M	1	–		2	2	–
18	M	1	–		2	2	–
19	M	1	–		2	1	+
20	M	1	+	Papilloma	2	2	–
21	M	1	–		2	2	–

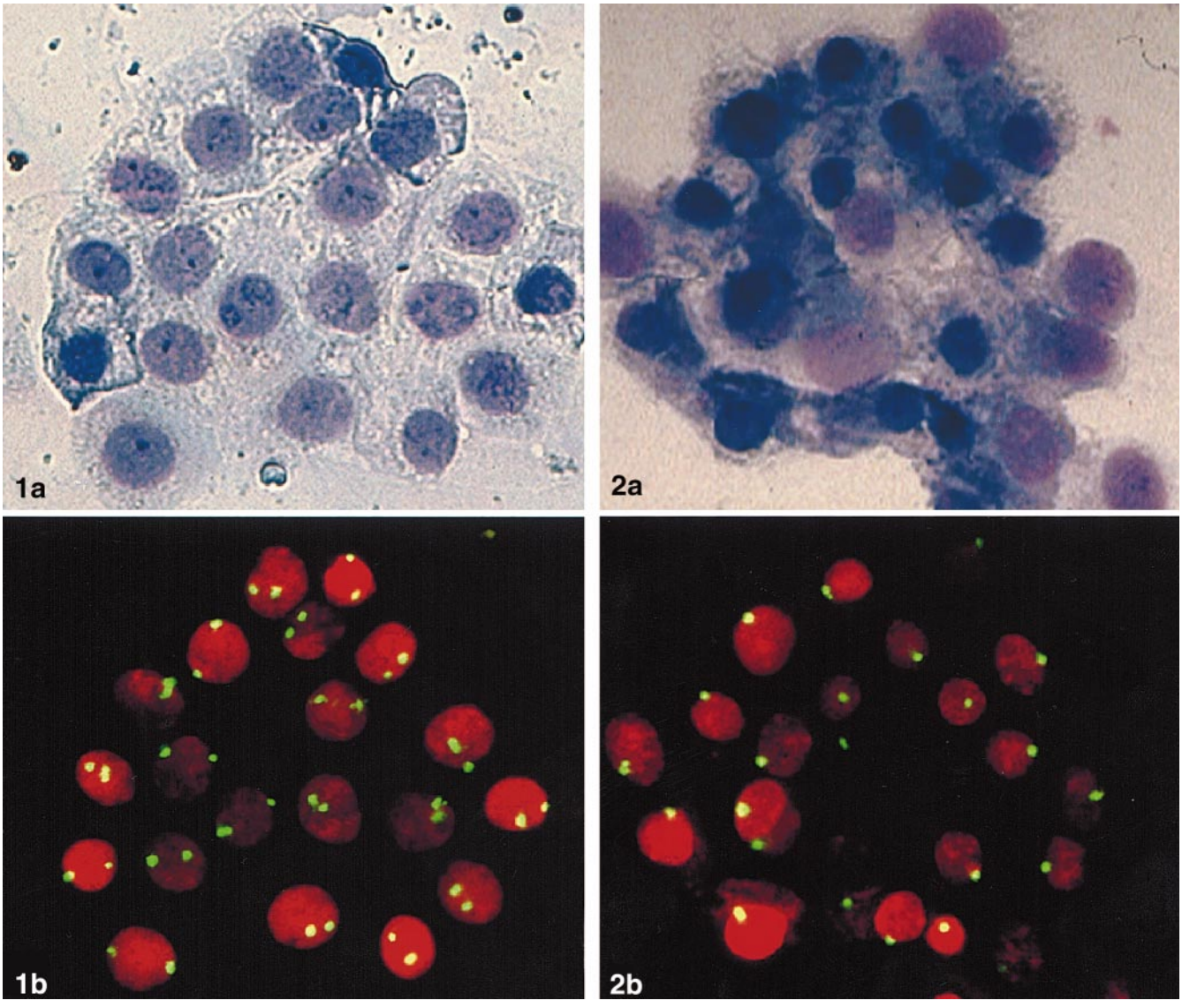

Fig. 1 **a** Giemsa stained cluster of the exfoliated cells from recurrence free case. **b** FISH on the same nuclei of the Giemsa stained cells. In each nucleus two signals were observed. In 95% of the nuclei examined two signals were observed

Fig. 2 **a** Giemsa stained cluster of the exfoliated cells of the patient with recurrent bladder tumor. **b** Same nuclei on FISH analysis using probe for chromosome 9. In the 85.5% nuclei, a single signal was observed. Cytology was class 3 and histology was TCC, G1, Ta (case 6)

in two cases (cases 5, 6). In case 5, tetrasomy 7 and tetrasomy 9 were observed. In case 6, during a previous hospitalization for transurethral resection of bladder tumor, FISH analysis showed the same aberration of monosomy 9 as was observed during this study. Case 4 showed normal a FISH score in spite of biopsy-proven recurrence resulting in a FISH false negative. Case 19 showed a positive chromosomal aberration but this turned out to be a false positive. Overall, the specificity of FISH for the detection of biopsy proven bladder cancer is 93.7% and sensitivity is 80%.

Discussion

Among highly differentiated TCCs of the bladder, high rates of false negative results from urine cytology have been reported, stressing the need for additional diagnostic procedures. Intensive work has been done by Tribukait [8] on the usefulness of quantitative measurements of nuclear DNA of bladder cancer tissue using flow cytometry(FCM). However, ploidy analysis of exfoliated cells in the urine using FCM is not an effective solution because the majority of highly differentiated TCCs of bladder show the same diploid pattern as seen in normal cells [1]. In this preliminary study, we applied the FISH technique to detect individual numerical chromosomal aberrations of exfoliated cells obtained from patients who had previously received treatment for superficial bladder cancer. We evaluated the value of FISH in the diagnosis of bladder cancer especially for the early detection of recurrence during the follow-up after a definitive treatment.

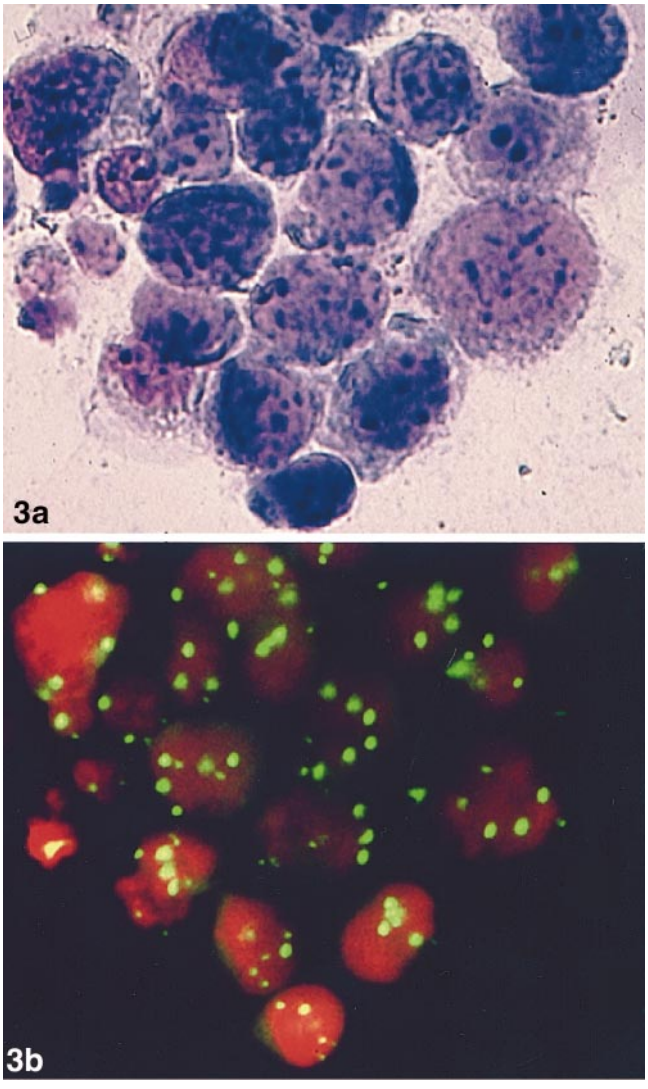

Fig. 3 **a** Giemsa stained cluster of the exfoliated cells of the patient with recurrent bladder tumor. **b** Same nuclei on FISH analysis using probe for chromosome 7. In the 36.0% nuclei 6 signals were observed. Cytology was class 5 and histology was TCC, G3, Tis (case 2)

A bladder wash contains inflammatory, squamous and normal transitional cells in addition to cancer cells. To detect cancer cells we selected clusters of transitional cells in Giemsa-stained smears and analyzed the chromosomal copy number of the selected cluster of transitional cells. In high grade TCCs of the bladder, cancer cells tend to dissociate in the bladder wash but we found we could still select clusters of such cells.

There is growing evidence for a tumor suppresser gene(s) on chromosome 9. Restriction fragment length polymorphism (RFLP) studies have revealed the frequent loss of heterozygosity (LOH) on both the long and short arms of chromosome 9. Heterozygosity on chromosome 9 is thought to be an early event in tumorigenesis of bladder cancer [9, 10, 11]. Our previous FISH study on 35 TCCs using satellite DNA probes for chromosomes 1, 7, 9, 11, 17 and Y revealed a relatively high rate of chromosome 9 deletion (32.4%) [4]. Numerical aberrations of chromosome 7 were rare in diploid cases and the copy number of chromosome 7 correlated most closely with the DNA index. In this study, we used the copy number of chromosome 7 as an indicator of ploidy, and the copy number of chromosome 9 as an indicator of deletion.

In case 6, monosomy 9 and disomy 7 were observed. We assume this TCC is diploid and that it was difficult to detect by FCM. In case 19, FISH revealed monosomy 9 but cystoscopically there were no abnormal findings. Intravenous urography was also normal. Although this case was regarded as FISH false positive, we believe this patient either has clinically undetectable TCC or has a high probability of forming tumors over time. This patient is now under close observation.

In this preliminary study, we used pericentromeric probes to detect numerical chromosomal aberrations. More precise FISH analysis, targeting the common deleted lesions on chromosome 9, 9p21–22 or 9q34, would significantly increase sensitivity [2, 7]. FISH is a relatively simple technique and more objective than conventional cytologic analysis. In the near future its clinical application in addition to or as an alternative for urine cytology should be possible. Further studies with a large number of cases and a comparative study with conventional diagnostic procedures should be performed. Sociomedical aspects including costs, work-load and the essential equipment should also be taken into consideration.

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