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Bladder Cancer

Clinical Utility of Fluorescent *in situ* Hybridization for the Surveillance of Bladder Cancer Patients Treated with Bacillus Calmette-Guérin Therapy

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Article info

Abstract

Objective: To evaluate the use of a multiprobe fluorescent *in situ* hybridization (FISH) assay for determining the response of patients with high-risk superficial bladder tumour (HRSBT) to bacillus Calmette-Guérin (BCG) therapy.
Methods: Bladder washing specimens from 65 HRSBT patients collected before and after BCG therapy were analyzed by FISH. Labelled probes for chromosomes 3, 7, 9, and 17 were used to assess chromosomal abnormalities indicative of malignancy.
Results: Fifty-five of 65 (85%) patients had a positive pre-BCG FISH result; 29 (45%) patients had a positive and 36 (55%) had a negative post-BCG FISH result. Patients with a positive post-BCG FISH status had a 2.7 times higher risk for tumour recurrence than patients with a negative post-BCG FISH status ($p = 0.017$; 95% CI: 1.18–6.15). In addition, patients who maintained a positive FISH status before and after BCG therapy had a risk for tumour recurrence 2.96 times higher than patients whose FISH result changed from positive to negative after BCG ($p = 0.02$; 95% CI: 1.17–7.54). On the other hand, there was no significant difference between the risk for tumour progression in patients with a positive versus a negative post-BCG FISH result ($p = 0.49$).
Conclusions: The high percentage of positive pre-BCG FISH results suggests the need for adjuvant therapy in patients with HRSBT after the initial transurethral resection. In addition, patients with a positive post-BCG FISH result were more likely to relapse after therapy. Thus, FISH appears to be useful for the surveillance of patients with HRSBT following BCG therapy.
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1. Introduction

High-risk superficial bladder tumours (HRSBTs; T1, G3, multifocal or highly recurrent, carcinoma in situ [CIS]) are associated with a high recurrence and progression rate. The established initial treatment for these tumours is transurethral resection (TUR) with adjuvant bacillus Calmette-Guérin (BCG) immunotherapy [1]. However, despite this combined therapy, a high proportion of these tumours recurs or progresses to muscle-invasive disease [2]. The risk of failure of intravesical BCG may be greater than 50% in long-term follow-up, and BCG immunotherapy it is not exempt of side effects [3]. Therefore, predicting the response to BCG treatment could allow us to identify patients who are candidates for alternative therapies before recurrence or progression to muscle-invasive disease have occurred.

The UroVysion FISH assay is a multitarget test that may be performed on urine cells and detects up to four chromosomal aberrations frequently associated with bladder cancer [4]. These alterations, consisting of aneuploidy of chromosomes 3, 7, and 17 and deletion of the 9p21 locus, have been reported to have higher sensitivity than urinary cytology for detecting urothelial carcinoma in urine or bladder washing specimens [4–7]. In addition, it has been suggested that this test may be useful for the detection of tumour recurrences before they are visible by cystoscopy [8]. In this study, we evaluate the use of the multiprobe FISH assay to predict the response of patients with HRSBT to BCG therapy.

2. Methods

2.1. Patient population

Sixty-five consecutive patients (8 females, 57 males; mean age: 70 yr; range: 33–86 yr) receiving BCG therapy between September 2003 and October 2004 were enrolled in this study. The hospital ethics committee approved this study, and all patients provided their informed consent before being enrolled.

Inclusion criteria for BCG therapy were primary or relapsing high-grade (HG) superficial bladder tumours with or without associated CIS (18 and 24 cases, respectively), low-grade (LG) superficial bladder tumours with associated CIS (five cases), multifocal LG superficial tumours (two cases), multirecurrent tumours (five cases), and isolated Tis (11 cases; Table 1) [9,10]. All patients received at least one cycle of BCG instillation, which consisted of six weekly instillations of endovesical BCG (81 mg Connaught strain BCG). In seven patients the samples were obtained when a second cycle of BCG instillations was performed. Two patients received fewer than three additional BCG doses after 3 mo of the first cycle, but none of them finished the maintenance schedule (one Tis and one T1 HG).

Tumour recurrence was defined as a histologically proven tumour after bladder biopsies or TUR, and progression was defined as the development of muscle-invasive disease. In patients who did not have recurrence or progression, follow-up was recorded as the number of months from the initial TUR to the last patient observation. In patients with recurrence or

Table 1 – Summary of FISH results, recurrences, and muscle progressions according to the pathological classification of the tumours and the inclusion criteria for BCG therapy

Inclusion criteria for BCG therapy	No. patients	Tumour stage and grade	No. positive FISH results		No. tumour recurrences	Muscle progression
			Pre-BCG	Post-BCG		
Superficial LG tumours with associated CIS	3	Ta LG + CIS	2	2	2	0
	1	T1 LG + CIS	0	0	1	0
	1	Tx LG + CIS	1	0	0	0
Superficial HG tumours	8	Ta HG	8	3	3	1
	11	T1 HG	9	4	4	2
	5	Tx HG	4	4	3	0
Superficial HG tumours with associated CIS	7	Ta HG + CIS	6	2	3	1
	9	T1 HG + CIS	8	4	3	1
	2	Tx HG + CIS	2	1	0	0
Isolated Tis	11	Tis	10	5	3	1
Multirecurrent tumours	2	Ta LG	2	2	1	0
	2	Tx LG	1	0	0	0
	1	Tx Gx	1	1	0	0
Multifocal tumours	1	Ta LG	0	0	0	0
	1	T1 LG	1	1	1	0

FISH, fluorescent in situ hybridization; BCG, bacillus Calmette-Guérin; LG, low grade; HG, high grade; CIS, carcinoma in situ.

progression, follow-up was recorded until the date of the event.

The clinical follow-up after the treatment consisted of cystoscopy and bladder washing cytology every 3 mo for the first 2 yr and every 6 mo thereafter. Selected patients with previous CIS also underwent multiple bladder cold-cup biopsies after BCG.

2.2. FISH analysis

Bladder washing specimens (50–300 ml) were collected just prior to the first BCG instillation (pre-BCG samples) and between 2 and 4 mo (mean: 2.9 mo) following the last BCG instillation in most cases (post-BCG samples). In six cases, however, samples were collected 5 mo after the last BCG instillation and in five cases after 6 mo because the patients failed to attend the appointment or collection was delayed for unrelated medical reasons. Samples were processed within 24 h after they were collected or were kept at 4 °C in 2% CarboWax solution until processed. Cells from bladder washings were processed and hybridized with the multitarget, multicolour FISH test UroVysion (Vysis Inc., Downers Grove, IL, USA) as described in Marín-Aguilera et al. [6,7].

Samples were evaluated by two observers blinded to the cytology, cystoscopy, and/or biopsy results. Scanning of the slides was performed considering cytologically atypical nuclei suggestive of malignancy (large nuclear size, irregular nuclear shape, patchy and often lighter nuclear DAPI staining). The criteria for being FISH positive were those suggested for the detection of urothelial carcinoma by Halling et al. [11]. When one of the criteria was met, the counting process was stopped. If none of the criteria for positive FISH was met, at least 100 selected nuclei were scored.

2.3. Statistical analysis

Cox proportional-hazards regression was used to determine the relative risk of recurrence and progression, at any time, for the patients according to their pre- and/or post-BCG FISH results. The cumulative distribution of the recurrence-free survival in patients with positive and negative post-BCG FISH results was estimated according to the Kaplan-Meier method. For all statistical tests, the significance level was set at $p < 0.05$.

3. Results

A total of 130 bladder washings were obtained from 65 patients who received BCG therapy (Table 1). During follow-up, 41 patients did not have recurrence or progression between 8.9 to 26.6 mo (mean: 16.4 mo) after the initial TUR, whereas 24 patients had tumour recurrence between 5.6 and 18.6 mo (mean: 11.6 mo) after the initial TUR. Of these patients with tumour recurrence, six presented a muscle-invasive carcinoma within a mean surveillance period of 13.3 mo (range: 9.8–18.6 mo) from the initial TUR. These six patients and three patients that presented a persistent CIS recurrence (Tis,

Ta HG + CIS and T1a HG + CIS) underwent cystectomies. Six HG superficial tumours, seven LG superficial tumours, and two Gx superficial tumours were also found within recurrent cases (Table 2). Additionally, four of the patients with corroborated tumour recurrence underwent a second cycle of BCG therapy (two Tis, one T1 HG, and one T1 HG + CIS). Two patients died during the follow-up period due to causes unrelated to the urothelial carcinoma, and four patients dropped out during the surveillance period.

Tables 3 and 4 show the pre- and post-BCG FISH results of the 65 patients. Ten patients (15%) had negative pre-BCG FISH result; of these, six also had a negative post-BCG FISH result and four had a positive post-BCG FISH result. Fifty-five patients (85%) had a positive pre-BCG FISH result; of these 25 also had a positive post-BCG FISH result and 30 had a negative post-BCG FISH result.

No association was found between pre-BCG FISH results and the risk of tumour recurrence ($p = 0.44$) or progression to muscle-invasive disease ($p = 0.97$; Table 3). In contrast, a significant association was identified between post-BCG FISH results and the risk of tumour recurrence ($p = 0.017$). Patients with a positive post-BCG FISH result had a 2.7 times greater risk of tumour recurrence than patients with a negative post-BCG FISH result (95% CI: 1.18–6.15; Table 3). On the other hand, six of the 24 patients with recurrent tumour presented a muscle-invasive carcinoma. Three of them had negative and three positive post-BCG FISH results (Tables 2 and 3). Hence, no association could be found between a post-BCG FISH result and the risk for tumour progression ($p = 0.49$).

Moreover, when analyzing the changes in FISH status before and after BCG therapy, we found that those patients with a positive pre- and post-BCG FISH result had a risk of tumour recurrence 2.96 times higher than those whose FISH result changed from positive to negative after BCG therapy ($p = 0.02$; 95% CI: 1.17–7.54; Table 4).

The actuarial curve for tumour recurrence using the Kapplan-Meier method (Fig. 1) showed differences in the percentage of recurrences and time to tumour recurrence between patients with positive and negative post-BCG FISH results ($p = 0.015$). Only 33% of the patients with a positive post-BCG FISH status remained free of recurrence through the study period, whereas 66% of the patients with a negative post-BCG FISH status remained recurrence free. On the other hand, patients with positive versus negative post-BCG FISH results had different mean times of recurrence: 17.1 mo (95% CI: 14.1–20.1) and 19.4 mo (95% CI: 14.1–20.1), respectively.

Table 2 – Summary of the relapsed patients' clinical evolution and FISH results before and after BCG therapy

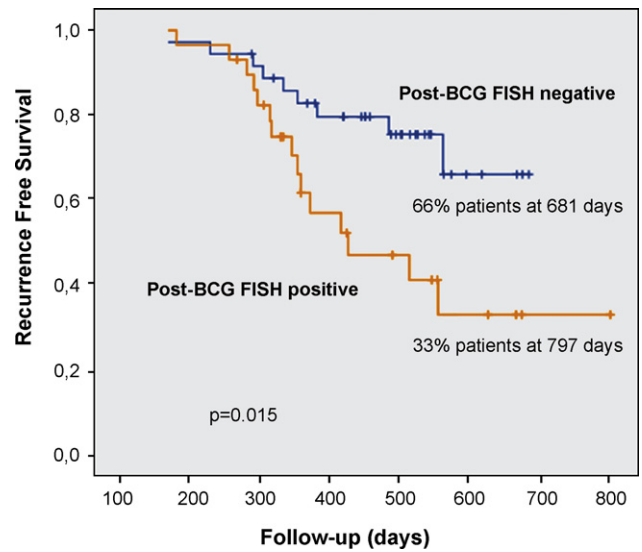| Patients with tumour recurrence	Pre-BCG therapy				Time to control after BCG therapy (d)	Control after BCG therapy		Time to recurrence after BCG therapy (mo)	TUR for recurrence			Muscle-invasive disease
	Tumour stage	Tumour grade	Associated CIS	FISH result		Clinical controls result[*]	FISH result		Tumour stage	Tumour grade	Associated CIS	
1	Ta	high	yes	positive	134	negative	negative	16.20	T2	high	no	yes
2	T1a	high	yes	positive	54	positive (Ct)	negative	13.67	Tis	high	no	no
3	Ta	low	yes	positive	6	negative	negative	7.07	Tx	Gx	no	no
4	Tx	high	no	positive	73	positive (C)	positive	6.83	Ta	high	no	no
5	T1	high	yes	positive	74	positive (Ct)	positive	8.23	Tis	high	no	no
6	Ta	high	no	positive	38	negative	positive	10.73	Tx	low	no	no
7	Tx	high	no	positive	68	negative (T)	positive	15.00	Ta	low	no	no
8	Ta (MF)	high	yes	negative	60	positive (B + C) + Susp. (Ct)	negative	2.00	Tx	Gx	no	no
9	Ta	high	no	positive	54	positive (C)	positive	9.47	Ta	high	no	no
10	T1c	high	no	negative	119	negative	positive	7.57	T2	high	no	yes
11	Ta	high	yes	positive	81	negative	negative	8.67	Tx	low	no	no
12	Tx	high	no	negative	60	negative	positive	9.40	Tx	low	no	no
13	Tis	high	–	positive	51	negative	negative	6.83	Tx	high	no	no
14	Ta	low	yes	negative	185	negative	positive	16.07	Ta (MF)	high	no	no
15	T1	high	no	positive	71	positive (C)	negative	10.30	T2	high	no	yes
16	T1c (MF)	low	yes	negative	150	positive (T)	negative	5.00	Ta	low	no	no
17	T1	high	no	positive	92	positive (Ct) + Susp. (C)	positive	5.43	Ta	high	yes	no
18	Ta (MR)	low	no	positive	47	negative	positive	8.73	Ta	low	no	no
19	Tis	high	–	positive	106	positive (Ct) + Susp. (C)	positive	11.43	T2a	high	no	yes
20	Ta	high	no	positive	141	negative	negative	7.77	T2	high	no	yes
21	T1	high	yes	positive	157	positive (C)	positive	7.23	T2	high	no	yes
22	Tis	high	–	positive	74	negative	positive	7.47	Tis	high	no	no
23	T1	high	no	positive	67	positive (C)	positive	3.60	T1a	high	yes	no
24	T1 (MF)	low	no	positive	102	positive (C)	positive	6.03	Ta	low	no	no

[*] Results obtained in the clinical surveillance protocol performed on patients after BCG therapy. In our institution, this consists in cystoscopy and bladder washing cytology every 3 mo for the first 2 yr and every 6 mo thereafter. Selected patients with previous CIS undergo multiple bladder cold-cup biopsies after BCG.

FISH, fluorescent in situ hybridization; BCG, Bacillus Calmette-Guérin; MF, multifocal; MR, multirecurrent; Ct, cytology; B, biopsy; C, cystoscopy; TUR, transurethral resection.

Table 3 – Recurrence and progression according to pre- and post-BCG FISH result

FISH status	Initial no. patients	No. patients with recurrence (%)	Follow-up[a] (mo)	Cox proportional HR (95% CI)	p value (log rank test)	No. patients with progression (%)	Follow-up[a] (mo)	Cox proportional HR (95% CI)	p value (log rank test)
Pre-BCG therapy									
Negative	10	5 (50%)	10.5 (5.6–18.4)	1 (reference)	0.44	1(10%)	14	1 (reference)	0.97
Positive	55	19 (35%)	11.87 (3–18.6)	0.67 (0.25–1.78)		5 (9%)	13.2 (9.8–18.6)	1.04 (0.12–8.84)	
Post-BCG therapy									
Negative	36	9 (25%)	11.5 (5.6–18.6)	1 (reference)	0.017*	3 (8%)	14.1 (11.1–18.6)	1 (reference)	0.49
Positive	29	15 (52%)	11.7 (3–18.4)	2.7 (1.18–6.15)		3 (10%)	12.6 (9.8–14)	1.76 (0.36– 8.70)	

HR, hazard ratio.
[a] Mean and range.
* Significant, $p < 0.05$.

Fig. 1 – Kaplan-Meier curves comparing recurrence of patients with positive versus negative post-BCG FISH results.

The FISH test performed after BCG therapy was unable to predict the tumour recurrence in nine patients (false negatives), although five of them were not predicted to have recurrence based on our centre's current clinical protocol of surveillance after immunotherapy (combination of cytology with cystoscopy, multiple bladder biopsies, or TUR). These recurrences were clinically confirmed during the subsequent clinical controls. On the other hand, the post-BCG FISH result was positive in six patients with tumour recurrence who were undiagnosed by the clinical controls (Table 2).

All samples with positive FISH results, except one, fulfilled the same criterion of FISH positivity (identification of five or more nuclei with gains in two or more chromosomes 3, 7, or 17). The remaining sample was positive due to the presence of a homozygous deletion of 9p21 in more than 20% of the nuclei counted.

4. Discussion

Although BCG therapy has been widely accepted as the optimal treatment for HRSBT after TUR, a high rate of tumour recurrence and progression has been reported after this treatment [3]. In fact, Pycha et al. [12], who first reported the influence of topical instillation therapy on chromosomal aberration in bladder cancer, noted that numerical aberrations of some chromosomes remained persistent in most cases after this immunotherapy.

Table 4 – Recurrence according to combined pre- and post-BCG FISH result

FISH status Pre/ Post-BCG therapy		No. patients	No. patients with recurrence (%)	Follow-up[a] (mo)	Cox proportional HR (95% CI)	p value (log rank test)
Negative	Negative	6	2 (33%)	6.6 (5.6–7.6)	1 (reference)	0.61
	Positive	4	3 (75%)	13.2 (9.3–18.4)	1.60 (0.26–9.76)	
Positive	Negative	30	7 (23%)	12.8 (9.6–18.6)	1 (reference)	0.02[*]
	Positive	25	12 (48%)	11.3 (3–17)	2.96 (1.17–7.54)	

FISH, fluorescent *in situ* hybridization; BCG, Bacillus Calmette-Guérin; HR, hazard ratio.
[a] Mean and range.
[*] Significant, $p < 0.05$.

Significant efforts have been made to define clinically useful prognostic factors for the response to BCG therapy [13–16]. To date, however, none have been shown to accurately predict which patients will respond to BCG therapy or which will recur or progress. In this study, we evaluated the ability of a multiprobe FISH assay to determine the response of patients with HRSBT to BCG therapy.

The vast majority of HRSBT patients (55 of 65, 85%) had a positive FISH result after the initial TUR. This finding seems reasonable, as it has been reported that some tumour cells may remain in the bladder after initial TUR [17,18], but they are not visible at the time of the resection, especially in CIS cases. These cells will become evident during the surveillance period when they become morphologically abnormal enough to differentiate from adjacent normal urothelium [19]. However, FISH methodology has the ability to detect chromosomal abnormalities associated with malignant development before their phenotypic expression [20]. Those patients harbouring genetically altered cells are at high risk of tumour recurrence. Thus, the use of an adjuvant therapy in this population after the initial TUR seems mandatory.

Taking into account the rate of BCG failures [3], it becomes clear that BCG treatment does not always completely eradicate tumours [21]. Monitoring patients with the diagnostic combination of cystoscopy and urinary cytology after BCG can lead to an equivocal interpretation, as the therapy can produce reactive urothelial changes. In contrast, chromosomal integrity is not affected by BCG therapy, urinary tract infections, haematuria, or any instrumentation process [12,22], thus allowing for the interpretation of FISH results. In this study, patients with a positive FISH result after BCG therapy were more likely to relapse and tended to recur earlier than patients with a negative FISH result after BCG therapy (Fig. 1; Table 3). Moreover, patients with positive pre- and post-BCG FISH results had a greater risk of recurrence than patients whose FISH status changed from positive to negative after BCG treatment

(Table 4). These findings confirm those described previously [21] and suggest that FISH analysis can help to elucidate which patients are at high risk of recurrence after BCG therapy. In contrast to the previous study [21], we found no associations between FISH results and the risk of progression to muscle-invasive disease. Furthermore, we found no association between pre-BCG FISH results and the risk of BCG failure or recurrence. The larger number of cases reported in the present study and/ or differences in the criteria of recurrence could account for these differences.

Although we found nine false negative post-BCG FISH results, the number of false negative results after therapy using the combination of the current clinical techniques was higher (n = 11; Table 2). This finding reveals that, regardless of its high sensitivity, FISH depends on the presence of a sufficient number of malignant cells in the sample. The aggressive exfoliation of the vesical mucosa during BCG therapy, tumour cells that do not exfoliate, or low tumour burden could preclude the fulfilment of these criteria [23]. Furthermore, the lack of sensitivity of UroVysion in these cases could be explained by the fact that this test is limited to four chromosomes (3, 7, 9, 17), which are only some of those most frequently altered in bladder cancer [24].

Fourteen patients with positive post-BCG FISH results have not developed tumour recurrence in a mean surveillance period of 15.9 mo (range: 8.9–26.6 mo), which could be defined as a false positive finding of the FISH technique. Nevertheless, it could also be related to the temporal difference between the initialization of the carcinogenetic process and its phenotypic expression [12]. Hence, it is possible that the follow-up period was not sufficient to detect the clinical recurrence in some patients. In a meta-analysis of 1421 patients treated with BCG, 38.6% had a recurrence after a median follow-up of 26 mo [25]. In this study, because the nonrecurrent tumours from positive post-BCG FISH patients had a median follow-up of only 15 mo, we cannot rule out this possibility.

Numerous studies have investigated the pattern of chromosomal aberrations in bladder tumours. Those tumours with numerical aberrations of chromosomes 7 and 17 had a substantially higher recurrence and progression rate and were related to more aggressive tumour behaviour [26–28]. Because we analyzed tumours with a high risk of recurrence and progression, these reports are concordant with our results. We found that all positive FISH samples but one presented polysomies of chromosomes 3, 7, or 17. In contrast, we identified the homozygous deletion of 9p21 in more than 20% of the nuclei counted in only one sample. As originally described by Spruck et al. [29], this genetic alteration occurs early in the oncogenesis of bladder cancer. Thus, it is probably hidden in our samples due to the presence of HG tumours, which tend to have numerous and greatly variable chromosomal gains and losses.

Unfortunately, both cystoscopy and cytology, which are used routinely after BCG treatment, depend on subjective visible changes. Moreover, their diagnostic accuracy is overestimated by most urologists [4,8]. In contrast, the FISH technique offers a more objective assessment of a tumour's biological behaviour. Consequently, with the knowledge that FISH can detect chromosomal changes even months before conventional surveillance techniques and given the high likelihood of recurrence of BCG-treated patients, it would be advisable to use the FISH test as an adjuvant surveillance method for those selected HRSBT patients who had no evidence of recurrence by conventional clinical controls after BCG therapy. If the post-BCG FISH result is negative in these selected patients, it could be expected that the patient is at low risk of recurrence and may be controlled regularly according to the current European Association of Urology Guidelines on Bladder Cancer [30]. However, if the post-BCG FISH result is positive, the urologist should assure that this result is not due to an undiagnosed tumour. Although there is still no accepted protocol for surveillance of these patients, some authors recommend the exploration of the upper urinary tract by an imaging technique, repeat cystoscopy, and bladder biopsies [20].

5. Conclusions

Our results on FISH analysis before BCG therapy in patients with HRSBT confirmed the need for an adjuvant therapy in these patients after the initial TUR. Moreover, post-BCG FISH results suggest that FISH analysis can allow the urologist to differentiate those patients with HRSBT who are more likely to relapse after BCG therapy. Thus, FISH is likely to improve the conventional surveillance techniques used after BCG therapy. In this way, HRSBT patients could be monitored more carefully and treated more aggressively to prevent tumour relapse, progression, and metastasis.

Conflicts of interest

None of the authors has any financial arrangement with any company.

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