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European Association of Urology

Urothelial Cancer

Utility of Fluorescence *In Situ* Hybridization as a Non-invasive Technique in the Diagnosis of Upper Urinary Tract Urothelial Carcinoma

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Article info

Article history:
Accepted August 22, 2006
Published online ahead of print on September 8, 2006

Keywords:
Carcinoma
Transitional cell
In situ hybridization
Fluorescence
Pelvic neoplasms
Ureteral neoplasms
Urinary tract
Urine

Abstract

Objectives: To assess the clinical utility of a fluorescence *in situ* hybridization (FISH) assay as a non-invasive method for diagnosing and monitoring urothelial carcinoma (UC) in the upper urinary tract (UUT).
Methods: Urine specimens from 30 consecutive patients with UUT UC and 19 healthy controls were analyzed by means of cytology and FISH. For FISH analysis, labelled probes to chromosomes 3, 7, 9, and 17 were used to assess chromosomal abnormalities indicative of malignancy. Sensitivity and specificity of both techniques were determined and compared. The frequency of chromosomal aberrations of malignant cells from UUT was also determined.
Results: Overall sensitivity for FISH was significantly higher than the corresponding value for urine cytology (76.7% vs. 36%, respectively, $p = 0.0056$). Specificities for FISH and cytology were 94.7% and 100%, respectively (p = ns). The positive and negative predictive values for FISH were 95.8% and 72%, whereas for cytology they were 100% and 54%, respectively. Of the genetically altered nuclei counted, 67%, 54%, and 43% presented polysomy in chromosomes 3, 7, and 17, respectively, and 21% presented a homozygous deletion of chromosome 9.
Conclusions: FISH assay of chromosomes 3, 7, 9, and 17 performed on exfoliated cells from voided urine specimens has greater sensitivity than cytology for detecting UUT UC whilst maintaining a similar specificity. The non-invasive nature of this method and its higher sensitivity could contribute to improving the current diagnosis of UUT UC.

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0302-2838/$ – see back matter doi:10.1016/j.eururo.2006.08.045

1. Introduction

Urothelial carcinomas (UCs) in the upper urinary tract (UUT) represent around 5% of all urothelial tumours. Although the incidence of UUT UC after primary bladder cancer is low (0.7–4%) [1], the natural history of UUT tumours is characterized by a high risk (40–75%) of having recurrent bladder tumours [2]. Therefore, an accurate diagnosis of the tumours and long-term surveillance of the whole urinary tract are mandatory.

Diagnosis of UUT tumours currently relies on imaging techniques complemented with urine cytology and/or ureteroscopy. The main imaging techniques used are those that detect a filling defect, such as intravenous pyelography (IVP), retrograde pyelography (RGP), and/or computed tomography (CT). IVP, RGP, and CT should not be used as the sole diagnostic tool because of their low sensitivity in the detection of small tumours and the amount of causes, apart from carcinomas, of a filling defect in the UUT [3]. On the other hand, cytology has a low sensitivity for the detection of low-grade lesions, and ureteroscopy is an invasive method with poor sensitivity, especially in flat tumours [4].

Genetic abnormalities occur in initial stages of tumour development and are the primary determinants of neoplastic transformation. Detecting such genetic aberrations can assist in detection of early tumours, surveillance of the cancer patient, and risk assessment of tumoural progression. Multi-FISH (fluorescence *in situ* hybridization) is a cytogenetic-based technology that enables the analysis of multiple chromosomes in several cells. The FISH technique has been used as a diagnostic test for some time in many areas of hematologic cancers and is being increasingly used as a diagnostic test for solid tumours. Specifically, the combination of fluorescence probes for the detection of numerical aberrations of chromosomes 3, 7, 9, and 17 in exfoliated cells from voided urines has already been shown to have high sensitivity and specificity in the detection of bladder cancer [5–9]. Interestingly, this set of probes doubles the accuracy of urinary cytology in application to low-grade-stage bladder tumours [10], and has high sensitivity and specificity in detecting UC in cytologic equivocal urine samples [11]. Since cytogenetic studies have reported that abnormal karyotypes in UUT UC do not differ from those found in UC of the bladder [12], the aim of the present study was to evaluate the utility of FISH on chromosomes 3, 7, 9, and 17 as a non-invasive method for the diagnosis of UUT UC.

2. Methods

2.1. Patients and samples

Between June 2003 and March 2006, voided urines from 53 consecutive patients initially diagnosed with UUT UC were prospectively included in this study. Subsequently, 23 patients were excluded for different reasons (7 cases had simultaneous UC in the bladder, 7 cases had no histologically confirmed tumour, and 9 cases did not have enough cells to be evaluated by FISH). Finally, voided urine samples from 30 patients (25 males and 5 females; average: 66 yr; range: 30–86 yr) with histologically confirmed UUT UC (1 pTis, 1 pTa low grade (LG), 4 pTa high grade (HG), 2 pT1LG, 3 pT1HG, 5 pT2HG, 10 pT3HG, and 4 pTxLG) [13,14], and 19 voided urines from donors with no evidence of upper urinary tract disorders (8 males and 11 females; average: 36 yr; range: 21–58 yr) were studied. Twenty-one samples belonged to patients who underwent radical surgery (20 nephroureterectomies and 1 ureterectomy), and nine were from patients that followed endoscopic resection. Tumours were found in the renal pelvis in 16 patients, in the ureter in 9, and in both localizations in 5 patients. The hospital's ethics committee approved this study, and all patients and controls provided their informed consent before participating in this work.

Between 30 to 100 ml of voided urines were collected the day before treatment and divided into two aliquots to be analyzed by both cytology (25 urines from UUT UC patients and 19 from healthy controls) and FISH (30 urines from UUT UC patients and 19 from healthy controls) techniques. For cytology analysis, samples were processed on the same day they were obtained. For FISH analysis, samples were processed within 24 h after they were collected, or they were kept at 4 °C in 2% Carbowax solution until processed.

2.2. Cytology

Urine cytologies were performed according to Papanicolaus' staining and were evaluated by an expert pathologist (F.A.) blinded to the patient's clinical history. The results were either considered as positive, negative, or suspicious. Suspicious cytology was defined as those samples that contained cells with morphologies that could be clearly classified neither as tumoural cells nor as normal cells. For the calculation of sensitivity and specificity, suspicious and negative cytologies were pooled together because, in our institution, these patients are monitored more carefully but do not receive any curative therapy.

2.3. FISH analysis

Cells from voided urine were sedimented at 600 × *g* for 10 min. The cell pellet was washed with phosphate buffer saline, sedimented at 600 × *g* for 10 min, and fixed twice in 8 ml of Carnoy solution (3:1 [v/v] methanol:glacial acetic acid). The final pellet was stored in 1–1.5 ml of Carnoy solution at −20 °C. Approximately 30 μl of the cell pellet suspension was dropped onto a glass slide, and a region with the appropriate cellularity was selected. Slides were pretreated with the use of a FISH

pretreatment kit (Vysis Inc, Downers Grove, IL, USA) according to manufacturer's instructions. Then, they were hybridized with the multitarget, multicolor FISH test UroVysion (Vysis Inc, Downers Grove, IL, USA) with the use of a HYBrite (Abbott-Vysis) according to the manufacturer's instructions with minor modifications. Briefly, between 1.4 and 2.5 μl of the probe mix solution was applied over each target area, depending on the cell density. Probe mixture consisted of four directly labeled probes to the pericentromeric regions of chromosomes 3 (SpectrumRed), 7 (SpectrumGreen), 17 (SpectrumAqua), and to the locus 9p21 (SpectrumGold). After hybridization, slides were washed three times at 45 °C for 10 min in 50% formamide solution, once in 2× SSC for 10 min, and once more in 2× SSC/0,1%NP-40 for 5 min. Finally, nuclei were counterstained by adding 7 μl of 4,6-diamidino-2-phenylindole (DAPI II) in target areas. Slides were stored at −20 °C for at least 20 min before their evaluation.

The evaluation of the samples was carried out by two different observers blinded to the group of patients analyzed. Basically, scanning of the slides was performed by considering cytologically atypical nuclei suggestive of malignancy (big nuclear size, irregular nuclear shape, patchy and often lighter nuclear DAPI staining). The criteria for FISH positivity were those suggested by Halling et al. [7] for the detection of UC. Briefly, a sample was considered FISH-positive for urothelial cancer if at least one of the following criteria was met: (1) identification of five or more nuclei with gains in two or more different chromosomes (3, 7, or 17); (2) identification of 10 or more nuclei with the same polysomy in one chromosome (3, 7, or 17); or (3) observation of homozygous deletion of 9p21 in greater than 20% of the nuclei counted. When one of the above 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.

2.4. *Statistical analysis*

For the analysis of sensitivity, specificity, and positive/negative predictive values of FISH and cytology, a chi-square test was used, and a 95% confidence interval was considered.

3. Results

Thirty urine specimens from patients with exclusively UUT UC were analyzed in this study. Cytology and FISH results for these samples are reported in Table 1. The overall sensitivity of FISH to detect UUT

Table 1 – Cytology and FISH results for patients with histologically confirmed UUT UC according to tumour stage and grade

Patient	Tumour stage	Tumour grade	FISH result	Cytology result
1	pTis	High	Positive	Suspicious
2	pTa	Low	Negative	Not performed
3	pTa	High	Negative	Negative
4	pTa	High	Negative	Negative
5	pTa	High	Positive	Suspicious
6	pTa	High	Positive	Positive
7	pT1	Low	Negative	Suspicious
8	pT1	Low	Negative	Suspicious
9	pT1	High	Negative	Suspicious
10	pT1	High	Positive	Positive
11	pT1	High	Positive	Suspicious
12	pT2	High	Positive	Suspicious
13	pT2	High	Positive	Not performed
14	pT2	High	Positive	Positive
15	pT2	High	Positive	Positive
16	pT2	High	Positive	Not performed
17	pT3	Low	Positive	Positive
18	pT3	High	Positive	Not performed
19	pT3	High	Positive	Suspicious
20	pT3	High	Positive	Positive
21	pT3	High	Positive	Not performed
22	pT3	High	Positive	Suspicious
23	pT3	High	Positive	Positive
24	pT3	High	Positive	Positive
25	pT3	High	Positive	Suspicious
26	pT3	High	Positive	Positive
27	pTx	Low	Negative	Suspicious
28	pTx	Low	Positive	Negative
29	pTx	Low	Positive	Negative
30	pTx	Low	Positive	Negative

FISH: fluorescence *in situ* hybridization; UC: urothelial carcinoma; UUT: upper urinary tract.

Table 2 – Comparison of sensitivity and specificity obtained from urine cytology and FISH analysis

Tumours	Sensitivity				*p* value
	Cytology		FISH		
	n positive/*n* total	Sensitivity (%)	*n* positive/*n* total	Sensititivity (%)	
By stage					
Non–muscle-invasive (pTis, pTa, pT1)	2/10	20	5/11	45.5	0.4387
Muscle-invasive (pT2, pT3)	7/11	63.6	15/15	100	0.0465*
pTx	0/4	0	3/4	75	0.1441
By grade					
Low grade	1/7	14.3	4/8	50	0.3606
High grade	8/18	44.4	19/22	86.4	0.0131*
Total	9/25	36	23/30	76.7	0.0056*
Controls	**Specificity**				***p* value**
	Cytology		FISH		
	n positive/*n* total	Specificity (%)	*n* positive/*n* total	Specificity (%)	
Healthy controls	19/19	100%	18/19	94,70%	0.9935

FISH: fluorescence *in situ* hybridization.
* Significant, $p < 0.05$.

UC was 76.7%, whereas that obtained by cytology was 36% ($p = 0.0056$). Cytology and FISH sensitivities detecting non–muscle-invasive tumours were 20% and 45.5%, respectively ($p = 0.4387$), whereas for muscle-invasive tumours they were 63.6% and 100%, respectively ($p = 0.0465$). Sensitivities of cytology and FISH by grade were 14.3% versus 50% for LG, and 44.4% versus 86.4% for HG tumours, respectively (Table 2).

Interestingly, six of seven false-negative samples by FISH were from patients with non–muscle-invasive tumours (one pTaLG, two pTaHG, two pT1LG, and one pT1HG) and one from pTxLG. Six of these samples were also analyzed by cytology, and rendered a negative and a suspicious result in two and four cases, respectively. It is of note that all samples positive by cytology were also positive by FISH (Table 1).

Among healthy volunteers without UC, the specificity of cytology and FISH was 100% (19 of 19) and 95% (18 of 19), respectively ($p = 0.9935$; Table 2). There was one false-positive result by FISH and one sample with suspicious result by cytology. These samples were obtained from two people (aged 30 and 27 yr, respectively) who were verified as not having a UUT disorder by an imaging technique. Both controls were nonsmokers and without history of any urologic disease.

The positive and negative predictive values of cytology for UUT tumours were 100% and 54.3%, respectively, and for FISH, 95.8% and 72%, respectively. Cytologic examination gave a suspicious result in 44% (11 of 25) of studied samples and, interestingly, the FISH technique was able to confirm the presence of tumour in 64% (7 of 11) of them. The sensitivity of FISH in each staged group of suspicious samples is shown in Table 3.

The most common FISH criterion found in the positive urine samples was the gain of two or more chromosomes in five or more urinary cells (21 of 23; 91.3%), whereas 8.7% (2 of 23) of cases were positive because 10 or more cells with a gain of a single chromosome were found (chromosome 3 in all the cases except in the false-positive control who had a

Table 3 – Sensitivity of FISH technique in suspicious cytologies

Tumours	Cases (*n*)	Suspicious cytologies (%)	FISH sensitivity of suspicious cytologies (%)
Non–muscle-invasive	10	60 (6/10)	50 (3/6)
Invasive	11	36.4 (4/11)	100 (4/4)
Tx	4	25 (1/4)	0 (0/1)
Total	25	44 (11/25)	64 (7/11)

FISH: fluorescence *in situ* hybridization.

gain in chromosome 7). Otherwise, no case was positive because of the homozygous deletion of 9p21 in 20% of the counted nuclei.

From the 30 samples studied by FISH, 554 genetic aberrant nuclei were counted, and 67%, 54%, and 43% of them presented polysomy in chromosomes 3, 7, and 17, respectively. Only 21% of genetically aberrant nuclei counted had the homozygous deletion of chromosome 9.

4. Discussion

UUT UC is not a frequent urologic cancer, but it presents clinical significance and some management difficulties. Moreover, UC in this location usually presents a high grade and stage [15], emphasizing the need for an early diagnosis and an effective treatment.

In the diagnosis of UUT UC, initial guidance with an imaging technique is imperative. In addition, confirmation by means of cytology or ureteroscopy is usually performed. Unfortunately, these complementary methods present some objections. On one hand, although cytologic examination of exfoliated cells in voided urine is an excellent tool for detecting high-grade urothelial tumours, with sensitivity as high as 95% and specificity higher than 90% [16], its role in the diagnosis of UUT UC is controversial because its sensitivity can diminish to 25% [17]. The collection of urine specimens by ureteral catheterization improves the sensitivity of this method from 35% to 88% [18] but produces discomfort to the patient. Nevertheless, whatever the method used for obtaining the urine, the lack of sensitivity persists, probably because of the difficult cytologic interpretation of the UUT cells [19]. Furthermore, it is possible to find transitional cells with equivocal morphology in urine samples, reflecting the limited value of this technique in distinguishing low-grade tumours from reactive urothelial changes [20]. On the other hand, endoscopy has a high sensitivity for detecting primary or recurrent UUT UC, but it is invasive, especially in the examination of more proximal locations of the urinary tract, and it has poor sensitivity in the detection carcinoma *in situ*.

A systematic review of urine markers for UC detection [21] showed that several urine-based tests have higher sensitivity than urine cytology for the detection of UC in the bladder although they have not been tested for the detection of UUT UC.

To our knowledge, this is the first study that reveals the utility of FISH as a non-invasive test in the detection of UUT UC. The sensitivity obtained by FISH on chromosomes 3, 7, 9, and 17 (76.7%) was significantly higher than the corresponding value for cytology (36%), as already described for the diagnosis of bladder cancer [22,23]. Even though it could be argued that the number of cases used to determine FISH and cytology sensitivities is different, comparison of the sensitivity results in only the 25 patients who had both tests performed still shows a significant difference ($p = 0.0106$). Previously, Lodde et al. [18] also reported the ImmunoCyt test to have higher sensitivity and specificity than cytology in the detection of UUT tumours although they used urine specimens obtained by ureteral catheterization [18].

Interestingly, an overall significant increase in sensitivity of FISH on chromosomes 3, 7, 9, and 17, with respect to cytology for the detection of UUT UC, has been obtained, while maintaining a similar specificity. It is of note that the specificity data of this study are in agreement with those obtained in studies with larger cohorts [5,6,9]. Although FISH sensitivity in superficial and low-grade tumours tends to be better than cytology, there is a lack of statistical difference that might be due to the low number of cases in these groups of tumours. On the other hand, our results show that FISH has a high predictive positive value (95.8%) that practically ensures the tumoural presence in the case of a positive result. In contrast, a negative FISH result correctly predicts the absence of tumour in 72% of cases. Probably, tumours misdiagnosed by FISH will be from patients with low-stage tumour because all negative FISH results in this study were from patients with non–muscle-invasive UUT UC.

With regard to chromosomal aberrations in this type of tumour, it is of note that most samples were positive by FISH because more than five nuclei with gains in two or more different chromosomes (3, 7, or 17) were found. In contrast, no case was positive because the only criteria fulfilled was the finding of homozygous deletion of 9p21 in greater than 20% of the nuclei counted. This aberration has been demonstrated to occur early in the development of urothelial tumours; thus, it is probably hidden in our samples because of the presence of high-grade, and consequently poorly differentiated, tumours in most of the patients studied. The high similarity between chromosomal abnormalities in UUT and bladder UC already described [12] and the results obtained in this study allow us to confirm the utility of voided urine for the detection of UC by FISH wherever the tumour is located in the urothelial tract. However, the main limitation of any urine-based method is the impossibility to detect the urothelial tumour's origin, which implies

the need to use complementary non-invasive imaging techniques.

In current clinical practice, a suspicious image of a UUT tumour obtained by IVP, RGP, or CT should be confirmed by urine cytology; when results are negative, an invasive endourologic technique must be performed. Because all samples positive by cytology were positive by FISH and the latter also has been demonstrated to improve the sensitivity of urine cytology, it seems reasonable to use FISH as an adjunct of cytology. Consequently, the combination of positive/suspicious results from an imaging technique with negative/suspicious cytology complemented by FISH would allow avoiding the use of any invasive technique in the diagnosis of UUT UC before treatment. However, in cases with a positive imaging technique and a negative cytology and FISH test, the use of endoscopic confirmation would be mandatory. On the other hand, because UUT UC tumours are generally multifocal throughout the urinary tract and conservative treatment could have been applied in selected patients as initial therapy [24], a careful follow-up of the urinary tract must be performed because of the high risk of recurrences. Although there is still no widely accepted protocol for surveillance of patients with primary UUT UC [25], methodologies currently used for this purpose are mainly based on image and invasive endoscopic strategies. The proven value of FISH in diagnosis allows undertaking studies to assess its value in the surveillance of recurrences of both bladder and UUT tumours.

5. Conclusions

In summary, in this study we show that FISH assay on chromosomes 3, 7, 9, and 17 improves the sensitivity of urine cytology in the diagnosis of UUT UC, while maintaining a similar specificity. This advantage would make FISH useful for corroborating first imaging suspicion of UUT UC with negative/suspicious cytologies, enabling a more accurate and non-invasive detection of this type of tumour.

Acknowledgements

This study was supported by a grant from the Spanish Urological Association (FIU 2004) and by a grant from Fondo de Investigaciones Sanitarias (FIS 04/2630). Mercedes Marín-Aguilera has a fellowship from IDIBAPS.

We thank Manel Fernández and Rosa Farré for their excellent technical assistance, and Dr Oliver for his help in the statistical analysis.

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Editorial Comment

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Marín-Aguilera et al. report their findings on the use of the UroVysion test to diagnose upper urinary tract urothelial carcinoma (UC). This commercial test uses fluorescence *in situ* hybridisation to detect copy number changes in four chromosomes commonly altered in bladder UC. In summary, the authors find that this test is more sensitive to cytology but still fails to detect one fourth of all tumours. The distribution of chromosomal alterations found is typical of bladder UC and supports the authors' hypothesis to use this test for the upper urinary tract. Evidence to date suggests that upper tract and bladder UCs are molecularly similar in most cases, with 10–15% of only upper tract UCs having microsatellite instability and frequent DNA methylation [1,2].

This report once again reveals the clinical limitations of current urinary tests for UC. It is not surprising that these tests fail to detect most tumours because any urologist knows UC (whether bladder or upper tract) is a heterogenous disease. At the molecular level superficial, well-differentiated and invasive, poorly-differentiated tumours are as similar as chalk and cheese [3]. To develop a test sensitive (and specific) enough to detect 99.9% of UCs (and therefore replace cystoscopy), we need a logical approach reflecting UC biology and a better molecular understanding [4]. For example, the hardest tumours to detect are well differentiated because they have few genetic alterations. One of the commonest changes found in these tumours is *FGFR3* mutation. The addition of *FGFR3* sequencing to chromosomal analysis increases urinalysis sensitivity to 90% [5], reflecting this logical tumour targeting. Still 10% of tumours had no genetic changes in this study, preventing their detection. To increase the sensitivity of urinary tests, we must deduce the molecular events in all UCs that drive them from normal cell control. Once we know this we can develop tests that may one day replace cystoscopy.

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