ORIGINAL ARTICLE

Liquid-based cytology as a tool for the performance of uCyt+™ and Urovysion® Multicolour-FISH in the detection of urothelial carcinoma

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Liquid-based cytology as a tool for the performance of uCyt+™ and Urovysion® Multicolour-FISH in the detection of urothelial carcinoma

The aim of the study was to assess the value of liquid-based urinary cytology as a tool to perform uCyt+ and Multicolour-FISH in patients under follow-up after urothelial cancer. Therefore, standard cytology was compared to liquid-based cytology with the addition of the uCyt+ test, which traces the three monoclonal antibodies M344, LDQ10 and 19A211 in exfoliated urothelial cells; and Multicolour-FISH (including centromere-specific probes for chromosomes 3, 7, 17 and a locus-specific probe for 9p21/p16) performed on thin-layer specimens. UCyt+ showed an overall sensitivity of 86.2% and cytology of 45.0%. Overall sensitivity of both the tests combined was 90%. Sensitivity of Multicolour-FISH was 96.4%. All conventional cytology diagnoses were confirmed by liquid-based cytology. Liquid-based cytology is a valid tool for the performance of adjunctive analyses, such as uCyt+ and Multicolour-FISH, on residual cellular material.

Keywords: urothelial cancer, uCyt+, Multicolour-FISH, liquid-based cytology

Introduction

In the last years, liquid-based cytology has been developed as an alternative to conventional cytological preparation methods. In particular, the ThinPrep® (TP) system has been widely accepted in non-gynaecological cytopreparation. Most comparative studies have shown that TP is equal to or better than conventional preparation with a sensitivity and specificity of over 90% in non-gynaecological specimens.[1] Furthermore, from the residual fluid further special studies, such as DNA analysis or immunhistochemical studies can be performed.[2]

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Cystoscopy is the most efficient method currently available to detect primary or recurrent urothelial cancer (UC) of the bladder, but it is invasive and causes significant discomfort to the patient. Furthermore, flat tumours or carcinoma *in situ* may be difficult to detect.[3] Urinary cytology is non-invasive and the gold standard in diagnosing high-grade lesions with a sensitivity up to 95% and a specificity close to 100%, but its sensitivity is low in grade (G) 1 tumours, the most common type of UC.[1–7]

Due to the limitations in sensitivity of cytology, sensitive and specific assays as adjuncts to urinary cytology for detection of UC have been developed. UCyt+™ detects cellular markers specific for bladder cancer in exfoliated cells of the transitional epithelium using three fluorescent monoclonal antibodies.[8] This test is highly sensitive for all grades of UC and improves significantly the detection of UC in

combination with cytology, profiting from the high specificity of the latter.[9–11]

The Urovysion® test is a multicolour, multitarget interphase fluorescence *in situ* hybridization (FISH) probe mix containing centromere probes for the chromosomes 3, 7 and 17 and a locus-specific probe to the band 9p21 (p16) which has been shown to be highly sensitive.[7,12,13] Although the performance of uCyt+™ on positive filtered specimens and of Urovysion® on sedimentation and cytospin slides is good, the preparation procedure is time-consuming and there is no cell material for eventually other analyses left after preparation.

The aim of the present study was to assess the value of liquid-based urinary cytology as a tool to perform further diagnostic and prognostic analyses such as uCyt+ and Multicolour-FISH in follow-up patients after superficial UC and patients with symptoms and signs suspicious for UC.

Patients and methods

Voided urines of 181 consecutive patients (mean age 67.03 years, range 32–83) were studied prospectively. 81 patients had symptoms suggestive of UC and 100 were under follow-up after complete transurethral resection (TUR) of UC at least 3 months previously.

40–80 ml of voided urine were collected from all patients and divided into two aliquots and evaluated by standard cytology performed by vacuum filtration, by liquid-based cytology (ThinPrep®; CYTYC Corp., Boxborough, MA, USA) and uCyt+™ (uCyt-kit; DiagnoCure Inc., Quebec, Canada). In 57 of 181 cases also a Multicolour-FISH (Urovysion®; Vysis Inc, Downers, IL, USA) was performed. Any cystoscopically suspicious lesion was biopsied or removed transurethrally. Histopathological classification was performed according to UICC criteria.[14]

Cytology

One part of the voided urines was used for vacuum filtered, standard urinary cytology (staining according to Papanicolaou, and haematoxylin and eosin). The remaining part of voided urine was used for the TP vial. Before preparing slides for uCyt+™ and Urovysion® a TP urinary cytology was prepared of every patient and stained according to Papanicolaou. Diagnostic results were categorized as previously published by Koss *et al.*[15] In short, specimens negative for malignancy or with atypia of any degree were categorized 'negative', and specimens considered suspicious or positive for malignancy as 'positive'.

uCyt+ staining

For the evaluation by uCyt+™ the second aliquot of fresh voided urine was added to previously prepared Falcon tubes containing 15 ml of Cytolyt (CYTYC Corp.) and sent to our laboratory. The sample was then centrifuged for 10 minutes at 2000 rpm and the cell pellet resuspended in the TP extragyn vial containing the PreservCyt solution (CYTYC Corp.). The vials were stored at 4 °C until thin-layer slide preparation (storage under these conditions is possible for up to one year) on Superfrost plus (Menzel-Gläser, Braunsch Weig, Germany) slides using the TP-System. Cells were fixed using a 50% Isopropanol spray. A positive and a negative control slide guaranteed a correct staining procedure. The number of cells on a slide served as a quality control measure. Slides containing less than 500 cells (urothelial and squamous cells) were excluded from the study.

For the uCyt+ procedure, slides were first pre-treated with ethanol 80%, 70% and 50%, distilled water and with Harris haematoxylin (Merck, Darmstadt, Germany), and with 4% acetic acid. After three washings in distilled water, cells were incubated with four drops of blocking solution for 15 minutes at room temperature in a closed humid chamber. The blocking solution was drained from the slides, which were incubated with the uCyt+™ cocktail for 1 hour at room temperature. Slides were then rinsed twice in phosphate-buffered saline (PBS) containing 0.5% Tween 20 and in pure PBS, and mounted with a cover glass. Negative and positive controls, blocking solution, and the antibody cocktails were provided in the uCyt™ kit.

Slides were read under a fluorescence microscope (Provis AX 70; Olympus, Milan, Italy) using filters for Fluorescein and Texas Red emission light detection. Red fluorescence showed cells positive for high-molecular-weight glycosylated carcinoembryogenic antigen, and green fluorescence cells positive for bladder cancer mucins. Samples were considered positive when they showed at least one green or one red fluorescent cell.

Urovysion® Multicolour-FISH

After pretreatment with 2 × sodium saline citrate (SSC) and 0.5 mg/ml Pepsin at 37 °C/0.01 N hydrochloric acid (HCl) in a waterbath, 1% formaldehyde

and PBS at room temperature the slides we dehydrated in 70%, 80% and 100% ethanol. After drying the slides, the Urovysion® probe mix was placed on the target and co-denaturation (5 minutes at 73 °C) and hybridization at 37 °C overnight were performed in the HYBrite™ oven (Vysis Inc.). The procedure was followed by a postwash using 0.4 × SSC/0.3% Nonidet (NP40) and 2 × SSC/0.1% NP40. Diamidinophenylindole (DAPI) II was used as counterstaining.

Slides were scored for hybridization signals on a cell-by-cell basis using an Olympus Provis AX 70 (Olympus) with a filter set including DAPI single bandpass (counterstain), aqua single bandpass (chromosome 17), yellow single bandpass (9p21 locus) and a red/green double bandpass (chromosome 3 and chromosome 7). Enumeration and evaluation of the FISH signals was done on target cells that appeared morphologically abnormal according to Bubendorf *et al.*[13] and the cut-off level was set at ≥4 aneusomic of 25 counted cells.

Sensitivity, specificity, negative and positive predictive value for cytology and uCyt+™ were calculated considering cystoscopy and histology as gold standard.

Results

Of the 181 patients 172 were evaluable. Eight specimens were rejected for uCyt+ because of the low number of cells (<500 cells per slide, urothelial and squamous cells) or because of intense granulocytosis and one patient was cytologically not evaluable. Five FISH samples were not evaluable because of insufficient urothelial cells and intense granulocytosis, and one among these five had a pTaG2 tumour.

Eighty of 181 patients had histologically verified UC of the urinary tract, and 101 patients were negative cystoscopically and/or histologically.

All conventional cytology diagnoses were confirmed by liquid-based cytology. Both, conventional and liquid-based cytology had a sensitivity of 45.0% (36 of 80) while uCyt+ had a sensitivity of 86.2% (69/80). Overall sensitivity of both the tests combined was 90.0% (72 of 80). Cytology missed 44 tumours (55.0%) and uCyt+ 11 (13.7%). Ten patients were negative for both (12.5%). All 10 patients had pTa G1 tumours. Specificity was 94.0%, 71.3% and 65.6% for cytology, uCyt+ and both tests together.

Sensitivity of Urovysion® was 96.4% (27 of 28) in the 57 analysed patients. Urovysion® missed one patient with a pTaG1 tumour. Specificity of Urovysion® was 46.4%.

Twenty four patients were negative for cytology but positive for uCyt+ and Multicolour-FISH. Ten of these 24 patients had pTa tumours, seven G1 and three G2.

Sensitivities, specificities and predictive values of positive and negative results of the three tests are given in Tables 1–3.

Discussion

In recent years, liquid-based cytology has emerged as an alternative to conventional cytology. Many laboratories have successfully applied this technique to body fluids (e.g. urines, pleural effusions), brushing samples and fine needle aspiration specimens. Most studies described equal or better results using the TP

Table 1. Sensitivity of cytology (conventional and LBC) and uCyt+ according to grade and stage of the tumours in 80 patients

$n = 80$	Cytology	uCyt+	Both
G1 ($n = 31$)	6.4% (2)	80.6% (25)	83.9% (26)
G2 ($n = 24$)	45.8% (11)	87.5% (21)	91.6% (22)
G3 ($n = 25$)	92% (23)	92% (23)	96% (24)
pTa ($n = 47$)	12.7% (6)	80.8% (38)	82.9% (39)
pT1 ($n = 13$)	92.3% (12)	92.3% (12)	100% (13)
≥pT2 ($n = 10$)	90% (9)	90% (9)	100% (10)
pTis ($n = 10$)	90% (9)	100% (10)	100% (10)

Table 2. Sensitivity of Urovysion Multicolour-FISH according to grade and stage of the tumours in 57 evaluable patients

$n = 28$	Multicolour-FISH
G1 ($n = 8$)	87.5% (7)
G2 ($n = 19$)	100% (19)
G3 ($n = 1$)	100% (1)
pTa ($n = 26$)	96.1% (25)
pT1 ($n = 1$)	100% (1)
pTis ($n = 1$)	100% (1)

Table 3. Overall sensitivity, specificity and predictive values of cytology (conventional and LBC) and uCyt+ in 181 patients and Multicolour-FISH in 57 patients

	Cytology	uCyt+	Both	Multi-FISH
Sensitivity	45.0%	86.2%	90%	96.4%
Specificity	94.0%	71.3%	65.6%	46.4%
PPV	85.7%	70.4%	69.2%	67.5%
NPV	68.5%	86.7%	90.0%	93.7%

PPV, positive predictive value; NPV, negative predictive value.

system compared with conventional specimens, and the residual material within the vial can be used for immunohistochemical or other special analyses.[7]

Given the limited sensitivity of standard cytology in low-grade UC, regular follow-up cystoscopies are still needed to monitor bladder cancer patients for recurrence or progression. Ancillary methods with improved sensitivity are therefore of high interest, as they could be used to select patients for follow-up schemes based on the individual risk of recurrence and progression, and reduce the number of control cystoscopies.[13]

At our institution, uCyt+™ and Urovysion® have been introduced for the monitoring of bladder cancer patients and TP is used routinely for gynaecological cytology. Therefore, we wanted to assess the value of liquid-based (TP) urinary cytology as a tool to perform further diagnostic and prognostic analyses such as uCyt+ and Multicolour-FISH.

UCyt+™ has been previously studied showing high sensitivity (85%–100%) for tumours of all grades.[8–11] Overall, its sensitivity was approximately two times higher than cytology. Sensitivity according to grade of UCs was much higher for uCyt+™ than for cytology in low-grade tumours and reached comparable values in high-grade tumours. This was confirmed in the present study. Overall sensitivity was 86.2% for uCyt+™ and reached 92% in high-grade tumours. Similar results were obtained in correlation with tumour stage. These high sensitivity values were not confirmed by Vriesma *et al.* who reported a sensitivity of only 50%.[16] The specificity of the uCyt+ test was reported previously to be lower than the specificity of cytology.[8–10] Our data showed a lower specificity of the uCyt+ than previously reported (65.6%) and an even lower specificity in patients under surveillance (63.8%). The negative predictive value of uCyt+ is comparable with those of previously studied urine-based tests[3,6] and higher than that for cytology. However, the combination of the uCyt+ test with cytology offers a negative predictive value (NPV) of 90%, which means that a patient with both tests negative has a reduced probability of having a tumour (10%). The presence of one green or one red cell appears to be the best cut-off level to obtain a high NPV and hence avoiding false-negative results.[8] Counting the positive cells could offer a further possibility of distinguishing patients with low and high probability of tumour. All conventional cytological diagnoses were confirmed by liquid-based cytology evaluated after immunofluorescence analysis.

Interphase FISH is increasingly utilized as an adjunctive method in the diagnosis of malignancies. Recently, a Multicolour-FISH assay consisting of four probes (CEP 3, 7, 17 and locus-specific probe 9p21) was developed and analysed on voided urines and bladder washings by several studies. Halling *et al.*[12] compared the Multicolour-FISH assay with cytology in patients under monitoring for tumour recurrence obtaining a higher sensitivity for FISH (81%) compared with cytology (58%) while maintaining high specificity. Bubendorf *et al.*[13] reported that FISH has almost twice the sensitivity of cytology in pTa and pT1 tumours (50.9% versus 29.8%). Most important, it identified 88% of invasive (pT1 and pT2–4) tumours on voided urine while sensitivity of cytology was 68% in the same tumour cohort. With the adjusted criteria for the definition of FISH-positive specimens, Bubendorf *et al.*[13] reported a three times higher sensitivity for FISH compared with cytology (73% versus 24%) in pTa tumours, and a 100% sensitivity in invasive tumours in contrast to the 58% sensitivity of cytology. Specificity was reported to be of 96%. In the present study, sensitivity of Urovysion® was 96.4%, higher than the results of Halling *et al.*[12] and Bubendorf *et al.*[13] in pTa, and comparable with the results in invasive tumours. Specificity was only 46.4% which is nearly the half of that described by the other studies. This might be explained by the fact that in the present study the Urovysion® test was performed only on patients under follow-up after UC, mostly classified as tumours at intermediate risk of recurrence while the previous studies[12,13] included patients without a history of urothelial carcinoma who were being evaluated for a variety of genitourinary signs and symptoms. This is also in accordance with a recent study of Sarosdy *et al.*[17] who describe a significant association of 'anticipatory positivities' with early tumour recurrence.

Skacel *et al.*[7] analysed Urovysion® Multicolour-FISH on TP slides of 20 voided urines concluding that TP slides, fresh and archival, can be used for Multicolour-FISH, allowing FISH technology more accessibility for routine use in cytology laboratories. These results are confirmed by the data of the present study. UCyt+ and FISH can be performed on TP material with high sensitivity and strong fluorescence, also if stored at room temperature over a longer period.

The clinical usefulness of a diagnostic test also depends on procedure time and technical expenditure. The uCyt+ test can be performed within 1.5 hours on specimens pre-stained with haematox-

ylin and 4% acetic acid for evaluation of the cytological morphology.[11] Cytology and uCyt+ can be performed on the same urine by the same cytologist. Multicolour-FISH needs a pre-treatment protocol of at most 70 minutes and hybridization takes between 45 minutes and 16 hours according to the protocol used.

Although liquid-based cytology is more expensive than conventional cytospin urinary cytology, it offers the possibility of further analyses. The cells can be stored in the PreservCyt-solution at 4 °C for up to 1 year and further important diagnostic and prognostic analyses can be performed at any time.

In case of positive uCyt+ patients can be forwarded directly to cystoscopy, while with both cytology and uCyt+ negative, cystoscopy may be avoided. Furthermore, in cases of interest a Multicolour-FISH could be performed out of the same TP vial using the same material as for cytology and uCyt+.

Conclusion

Using liquid-based urinary specimens further diagnostic and prognostic analyses such as uCyt+ and Urovysion® Multicolour-FISH may be performed on the residual material, in case of necessity also using stored material. It could be an adequate procedure for the screening of patients at risk for UC and the surveillance of patients under follow-up, avoiding superfluous cystoscopies.

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