Fluorescentna in situ hibridizacija kot neinvazivna tehnika odkrivanja raka mehurja v vzorcih urina

Non-invasive bladder cancer detection by fluorescent in situ hybridization on urine samples

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Ključne besede:
neoplazija mehurja, občutljivost in specifičnost, fluorescenčna in situ hibridizacija, genetski označevalci.

Key words:
bladder neoplasms; sensitivity and specificity; fluorescence in situ hybridization; genetic markers.

Članek prispel / Received
12.05.2009
Članek sprejet / Accepted
17.02.2010

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Izvleček

Namen: *Namen študije je bil ovrednotenje UroVysion FISH testa za odkrivanje celic raka mehurja pri uroloških bolnikih iz vzorcev urina.*
Metode: *Vzorce urina smo zbrali pred cistoskopijo. V primeru odkritega tumorja, je bila izvedena resekcija in histološka potrditev rakastega obolenja. Za odkrivanje rakastih celic smo uporabili metodo fluorescentne in situ hibridizacije (FISH) z uporabo DNA–sond, specifičnih za kromosome 3, 7 in 17 ter lokus–specifično sondo 9p21. Pregledanih je bilo vsaj 25 morfološko spremenjenih celic oz. vse prisotne celice na preparatu.*
Rezultati: *Od 179 pregledanih vzorcev, je bil en inficiran, v 35 (20%) pa ni bilo prisotnih celic. Statistično je bila metoda FISH ocenjena z občutljivostjo 76,2% (95% CI 52.8*

Abstract

Purpose: *To evaluate the diagnostic accuracy of the UroVysion FISH test for detecting transitional cell bladder cancer on a mixed sample of urological patients.*
Methods: *Urine samples were collected from patients before cystoscopy. In the case of tumor identification, transurethral resection and histological verification were performed. The fluorescent in situ hybridisation (FISH), using a commercial kit (UroVysion) containing hybridization probes for chromosomes 3, 7, 17, and 9p21, was used. At least 25 morphologically abnormal cells or all cells present on the slides were analyzed.*
Results: *Of 179 samples, 1 was infected and in 35 (20%), no cells were identified. Test sensitivity was 76.2% (95% CI 52.8–91.8) and specificity*

– 91.8) in specifičnostjo 93,6% (95% CI 88,6 – 96,9), kar je v primerjavi s podatki iz literature, dober rezultat. V večini primerov je šlo za številčne spremembe kromosomov. Histološko je bilo potrjenih 11,8% tumorjev.

Zaključek: *Metoda FISH je dokaj draga in zahteva dvodnevno obdelavo, kar je v primerjavi s citologijo ovira. Tako FISH kot citologija ne dajeta 100% odgovora, vendar velja metoda FISH za občutljivejšo metodo od citologije. Predvsem pa je prednost FISH v neinvazivnosti postopka, saj dobimo rezultat iz urina in se pogosto izognemo cistoskopiji.*

93.6% (95% CI 88.6–96.9); as compared to the data in the literature, this result was considered significant. Most tumors had numerical chromosome aberrations. The prevalence of histologically, which verified tumors, was 11.8%.

Conclusion: *Price and time–consuming procedures are major obstacles for use of the UroVysion FISH test. However, neither cytology nor UroVysion FISH are 100% specific. Higher sensitivity compared to cytology, which is evident not only in case of superficial, but also of invasive tumors (like T1) and its ability to provide numerical results, are advantages which may make UroVysion FISH test useful. Thus, this test has potential for future use.*

INTRODUCTION

Bladder cancer is among the ten most common cancers in adults. Due to the high recurrence rate up to 70% non–invasive tumor markers are necessary for patient monitoring. Better non–invasive tests are also necessary for initial diagnosis and even screening of high–risk populations. Among the many tests proposed for this purpose (UBC, BTA, Cyfra21–1, ImmunoCyt, NMP–22...), the UroVysion FISH test is unique as it does not depend on measuring biochemical methods and setting of an arbitrary level for a positive result. Instead this test directly identifies and counts transformed cells in urine samples, using multicolour fluorescence *in situ* hybridisation. In this study we investigated the diagnostic accuracy of UroVysion FISH test to identify transformed cells in voided urine specimens. The UroVysion FISH test can be used as a tool for detecting transitional cell bladder cancer.

MATERIAL AND METHODS

Participants

Urine samples from patients with a suspicion of bladder cancer were analyzed in the study. Specifically, the UroVysion FISH test was made available to practicing urologists in our institution. Use of the test was based on the urologists' discretion for cases where they suspected the results may help them in their clinical decision–making. Cells were isolated from urine prior to cytoscopy or biopsy and histological examination, which were performed within 3 weeks from urine sampling. All consecutive test samples, sent to the laboratory, were included in the present analysis.

Test method

A commercial kit (UroVysion) containing hybridization probes for chromosomes 3, 7, 17, and 9p21 was used according to the manufacturer's protocol (1). Briefly, urine samples (35 mL) were mixed in 2:1 (V:V) with Carbowax preservative (2% polyethylene glycol in 50% ethanol) and were transferred into 50 mL centrifuge tubes. After centrifugation, cells were treated with a hypotonic solution (PBS) and fixed in Carnoy fixative (methanol: acetic acid, 3:1), three times. Cell pellets were resuspended to an appropriate cell concentration (~ 100 cells per field). After drying, slides were incubated in 2X SSC for 5 minutes at 37°C. Protein digestion was performed in a pepsin buffer (10 mM HCl, 25 mg pepsin at 2500 – 3000 U/mg) for 10 minutes at 37 °C. Slides were then washed twice for 2 minutes in 2X SCC. Further fixation was carried out in 1% formaldehyde for 10 minutes. After two 2 minute washes in 2X SSC, slides were dehydrated in ethanol (70%, 90%, 100%).

Hybridization probe was prepared according to Vysis protocol and denatured at 73 °C for 10 minutes. Slides were denatured in 70% formamide/ 2X SSC at 73°C for 3 minutes. The probe mix was applied

to the slides and covered with 12 mm^2 plastic coverslips. Hybridization was performed in a humidified chamber at 37 °C overnight.

After removing the coverslip, slides were washed in 0.4X SSC/0.5% Tween at 73°C for 2 minutes and washed in 2X SSC/0.50% Tween at room temperature for 30 to 60 seconds. Before analysis, 10 µl DAPI II/antifade was added on the slide for visualization of nuclei. Slides were scored for hybridization signals using the Zeiss fluorescence microscope Axioplan 2.

At least 25 morphologically abnormal cells of all cells present on the slides were analyzed. A specimen was considered as FISH positive for bladder cancer if one of two criteria were met: 4 or more cells showing gains of more than one chromosome (3, 7 or 17) in 25 morphologically abnormal cells, or 12 or more out of the 25 cells having zero 9p21 signals (Fig.1).

Statistical methods

Test accuracy estimations were calculated using standard deviations (2,3). For confidence intervals calculation, Meta–Disc software was used (4).

RESULTS

Overall 179 samples were collected from 139 patients in the years 2005 to 2007. One sample was excluded due to a large number of bacteria present, and 178 samples were included in the analysis: 67 from females (48%) and 72 from males (52%).

In 35 of 178 samples, no cells were found (19.7% of all samples) – so called "empty" samples. Considering "empty" samples, they were significantly more often collected from male compared to female patients: 6 (17.1%) from females and 29 (82.9%) from males ($p<0.01$). Empty samples were considered as negative according to our previous discussion (5).

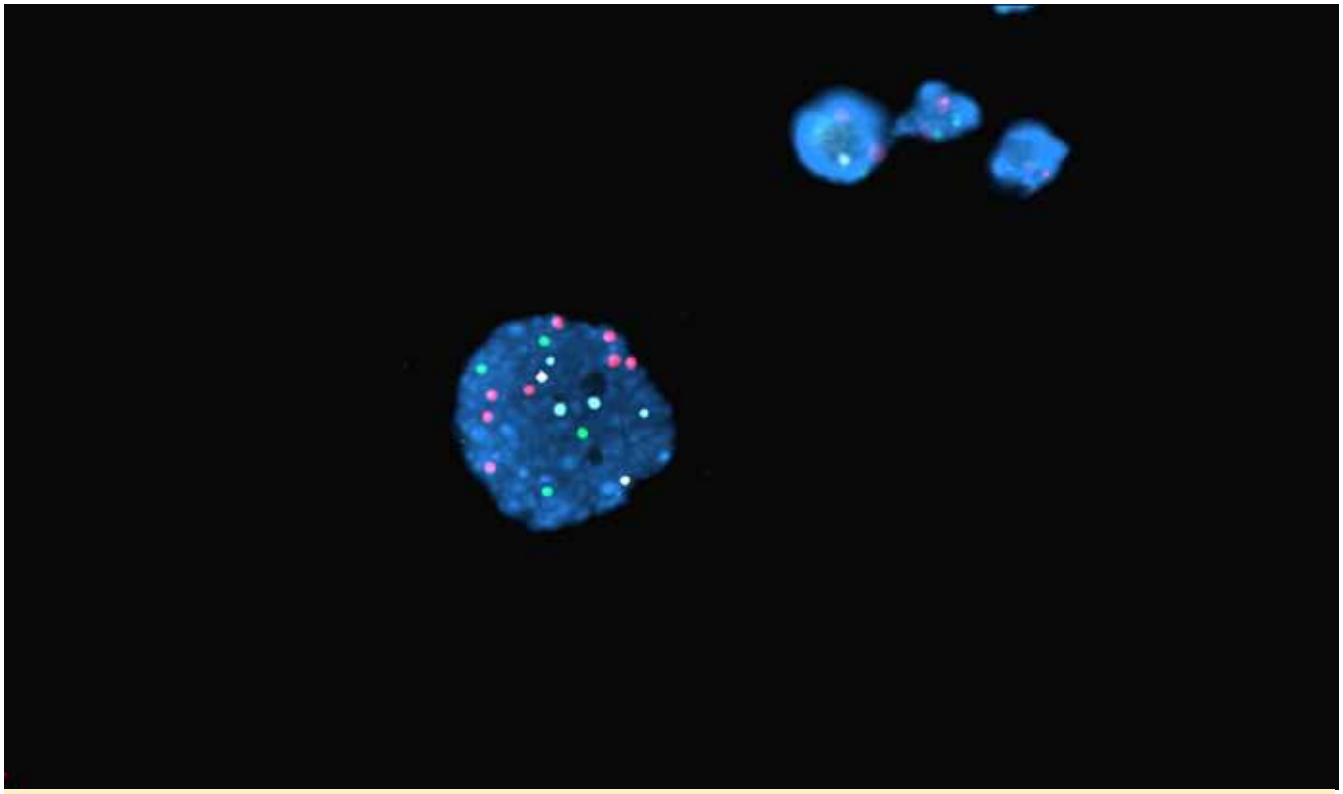

Figure 1. *Abnormal result observed in an aneusomic interphase cell obtained from a sample showing eight copies of chromosome 3 (red), four copies of chromosome 7 (green), four copies of chromosome 17 (aqua) and two copies of p16 gene (gold) after hybridization with the UroVysionTM Bladder Cancer Kit (UroVysion Kit).*

Prevalence of bladder cancer was 11.8% (21 histology positive). Among those, FISH detected 16 samples (sensitivity 76.2%, 95% CI 52.8–91.8) and there were 5 (23.8%) false negative samples. Among the negative samples, FISH correctly identified 147 samples (specificity 93.6%, 95% CI 88.6–96.9) and there were 10 (6.4%) false positives.

The results correlated with biopsy histological examination. The distribution of FISH positive cases after pathological classification corresponded to 7 low grade and 13 high grade tumors. Positive FISH results correlated with tumor stage in the following manner: 6 Ta, 4 T1, 6 T2 and 4 T3. Two of them had deletion of both copies 9p21, others had amplifications of all observed chromosomes.

Five cases were false negative; 4 of them were TaG1 and 1 was T1G1. One of those cases was positive after the second examination. The other 4 cases were once again tested after histopathological examination. Three of them were UroVysion positive and one case was negative with monosomies of all chromosomes tested.

Likelihood ratios for the overall group of samples were as follows: positive (LR+) 12.0 (95% CI 6.3–22.8) and negative (LR–) 0.25 (95% CI 0.12–0.55). Diagnostic odds ratio (OR) was 47 (95% CI 14–155).

When limiting analysis only to invasive tumors (excluding 6 superficial tumors), sensitivity increased to 85.7% (95% CI 57.2–98.2) and this improved the positive likelihood ratio to 13.5 (95% CI 7.1–25.5), the negative likelihood ratio to 0.15 (95% CI 0.042–0.55) and the diagnostic odds ratio to 88 (95% CI 17–449).

DISCUSSION

The UroVysion test introduced 6 years ago involves a very elaborate protocol, which is, at least at present, both very labor intensive and investigator dependent. Although its use of numerical criteria makes it more exact than cytology, the test is variable because the results of the test depend on the interpretation of the person who counts and examines the slides.

Since the introduction of the UroVysion test for detecting urothelial cancers in urine, its reported performance has varied. One of the reasons for the Urovysion FISH test performance variability may be the evolution of criteria, which determine a positive result. At the beginning, at least 5 cells with a gain of two or more chromosomes or at least 10 cells with a gain of a single chromosome, or homozygous deletion of 9p21 were considered positive (1). Some authors considered a positive sample which had 20% or more cells with gain of one chromosome or at least 40% of cells with deletion of 9p21(6). Another group counted all nuclei and accepted criteria for a positive result as 16% and 48% positive cells (7).

We favor the approach which takes advantage of visually selecting suspicious or abnormal nuclei (irregular, large) under DAPI staining and counting signals only in those cells. A very important factor for good results is when an experienced observer counts for a minimum number of abnormal cells, which are detected by screening and interpretation of FISH morphology on cells in interphase. It is also far less time consuming than counting all nuclei. This approach is at present also suggested by the manufacturer and adopted by the most recent trials.

Furthermore, it is vital that cases with a low number of available cells from voided urine samples are interpreted with caution. The evaluation of positive cells, which do not have the criteria for the FISH positive result, should be taken into account as borderline cases. This minimizes the false negative result and increasing the sensitivity of the test.

UroVysion FISH is a new test and its constant development and evaluation is evident from the above mentioned changing of criteria for a positive result. This is expected to continue, as studies which concentrate on follow–up cases have recently suggested to increase the criteria for a positive result from four

to at least 10 positive cells in order to decrease anticipatory positive (false negative) results (8).

Any test repetition improves sensitivity of diagnostic tests and UroVysion FISH is not an exception. In one of our false negative cases, a negative result was obtained after the first examination and positive after the second examination.

The sensitivity (76%) and specificity (93%) of our FISH results compare quite favorably to the results from the literature, which state 75% overall sensitivity and 85% specificity (9).

Compared to cytology, at least in our hospital, FISH is a more accurate test. From 21 FISH and histology positive cases, cytology was negative in 5 cases and there was not a single case found where cytology would be positive and the FISH test negative.

In clinical practice, we identified two scenarios, which illustrate practical points of UroVysion FISH use: a negative result in BCG treated patients and a "false positive" result. A negative UroVysion FISH result is very reassuring in patients after BCG therapy, with red–looking bladder mucosa and one unclear or undetermined cytology result.

Positive FISH may cause grave concern in patients, in whom no tumor can be confirmed histologically. Questions start to arise such as : "Is the tumor in the upper tract and are we not able to visualize it? Did random biopsy miss *in–situ* focus? Should we treat it and how?

The UroVysion FISH test brings in as much reassurance as concern to every–day practice. The method itself is still evolving. The criteria for a positive result is changing in the clinical setting, and its precise role in clinical algorithms is still in question. Although presently seen by some as just sometimes useful and quite expensive, positive UroVysion FISH tests tell us something we did not know before; it determines the presence and number of aneuploid cells in urine However the question still remains, "Do these cells arise from a settled tumor or are they just an indication of the presence of transformed urothelium or of successful elimination due to an organism's defense? Furthermore, are they clinically important?" The test can not tell and thus adds another uncertainty in the already complicated cancer equation. Some do not like these uncertainties and say "Life is more complicated than theories and test results."

UroVysion FISH is one of the "genetic" tests, which entered into practice of medicine, whether some are for it or against it. There are other tests used in clinical diagnosis, whose accuracy comes into questions. For example HPV testing in primary cervical cancer screening; it can seem too sensitive by detecting cells which could result from spontaneously regressive or irrelevant lesions, or seem insufficient by not detecting cells from lesions which simply do not possess the genetic test for the aberrations we are looking for (10).

Results of UroVysion FISH do not provide conclusive evidence for the presence or absence of urothelial cancer. However, both positive and negative test outcomes very often give important clues and also present strong modifiers of pre–to–post–test probabilities in formal diagnostic reasoning. Positive predictive values are around 5 for a mix of superficial and invasive tumors and more than 6 for only invasive tumors, and negative predictive values of 0.3 for all and less than 0.2 for invasive tumors.

Price and a time–consuming procedure are major obstacles for wider use of the UroVysion FISH test. Neither cytology nor UroVysion FISH are 100% specific (9,11,12). A higher sensitivity compared to cytology, which is evident not only for superficial, but also for invasive tumors (like T1) and its ability to provide numerical results, are advantages which may make the UroVysion FISH test useful in the future.

ACKNOWLEDGEMENTS

The authors acknowledge Primarius Karel Kisner, Head of Department of Urology, University Medical Centre Maribor, Maribor, Slovenia, for support and encouragement.

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