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ARTICLE

Detection of Bladder Cancer from the Urine using Fluorescence *in situ* Hybridization Technique

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The authors report on their first experiences with the UroVysion fluorescence *in situ* hybridization (FISH) kit developed for the detection of bladder cancer. This new non-invasive diagnostic application of the FISH technique in the field of urology was elaborated to replace cystoscopy. The special urine examination method detects genetic alterations of the urothelial cells found in the urine, using fluorescent direct-labeled DNA probes binding to the peri-centromeric regions of chromosomes 3, 7 and 17 as well as on the 9p21 locus. We aimed to evaluate the utility of UroVysion test in the light of the histological diagnosis. Urine samples from 43 bladder cancer patients and 12 patients with no or benign alterations were studied using a new application of FISH technique: the UroVysion reagent kit. The obtained FISH results were compared with the histological findings of the transurethral surgical resection specimens. The study rated the specificity and sensitivity of the technique 100% and 87%, respectively. Therefore, the technique could well fit into the diagnostic process of bladder carcinomas. Statistical analyses showed significant correlation between tumor progression and the severity of the genetic alterations detected by this FISH technique. Furthermore, positive correlation was found between tumor grade and the proportion of tumor cells showing genetic abnormality. The non-invasiveness, the robustness of evaluation and the high specificity/sensitivity are all in favor of this technique. The disadvantages are the higher costs of the technical background and the required future clinical studies to determine whether this technique can replace cystoscopy. (Pathology Oncology Research Vol 13, No 3, 187–194)

Key words: bladder cancer, cystoscopy, fluorescence in situ hybridization, molecular pathology

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Abbreviations: BTA test: Bard's bladder tumor antigen test, CCD: charge-coupled device, CDKN2A: cyclin-dependent kinase inhibitor 2A, CEP: chromosome enumeration probe, CT: computed tomography, DAPI: 4',6-diamidino-2-phenylindole, FDA: United States Food and Drug Administration, FISH: fluorescence in situ hybridization, K-W/DMC tests: Kruskal-Wallis / Dunn's multiple comparison tests, LSI: locus-specific identifier, MRI: magnetic resonance imaging, MW test: Mann-Whitney test, NMP22: nuclear matrix protein 22, PBS: phosphate-buffered saline, PCR: polymerase chain reaction, SSC: sodium chloride and sodium citrate solution, TPA: tissue polypeptide antigen, UroVysion test-positive cells: cells showing cytogenetic abnormalities listed in positivity criteria of UroVysion Bladder Cancer Recurrence Kit

Introduction

The urothelium is the epithelial lining of the uropoetic organs. It can be found interconnected from the renal cavity system via the ureters and the urinary bladder till the proximal third of the urethra in both males and females. Urinary bladder carcinoma counts as the most frequent malignant tumor of the urothelium. Considering histological type, 94% of these tumors are composed of transitional cells.[1, 2]

The most conspicuous symptom of bladder cancer is microscopic or macroscopic hematuria. Less frequent symptoms are difficult urination, frequent urination, therapy-resistant urinary tract infection. Infrequently, the medical checkup is initiated due to general symptoms such as weight loss or anemia. Nowadays, the disease is rarely diagnosed in such a late stage when metastatic symptoms, as bone pain or laborious breathing are present.

If the possibility of urinary bladder cancer emerges, small pelvic and abdominal ultrasound examination with a full bladder is the next step, which may show an intravesicular mass or consequential dilatation of the renal cavity system. The sensitivity of the ultrasound examination greatly depends on the quality of the apparatus and on how experienced the clinician is. It is not easy to recognize a small size tumor or one situated at the posterior wall. More sensitive radiological examinations, such as computed tomography (CT) or magnetic resonance imaging (MRI) are not justified because the invasive cystoscopy is imperative in clinical protocols for detecting the tumor.

The commonly used urine cytological examination is another examiner-dependent technique. However, it has the advantage of being non-invasive, although a big disadvantage is its doubtful sensitivity mostly notable in case of low-grade (well-differentiated) tumors.

Final, exact diagnosis can only be made by histological examination after the transurethral removal of the tumor or, depending on its size, from biopsy specimen.

Approximately 70% of primary tumors are stage pTa or pT1 superficial bladder carcinomas, 70% of which recur within the first year after surgical removal.[3] The regular, three monthly control examination includes cystoscopy.

There have been efforts to elaborate a good reproducible method which could be used instead of cystoscopy, by means of an appropriate, sensitive tumor marker or by increasing the sensitivity and specificity of urinary cytology.

Urinary cytology used for the detection of urinary tract tumors, particularly in case of early stage lesions, is many times not sensitive enough and, in addition, demands the high and specific qualification of the examiner. Hence, new diagnostic tools detecting DNA alterations of tumor cells are justified to become part of urological tumor diagnostics, besides morphologic criteria.[4-6]

The most frequent genetic alteration characteristic to malignant urothelial tumors is the partial or complete loss of chromosome 9, which can be observed as early as the onset of tumor formation. The p16 (CDKN2A) tumor suppressor gene found on the 9p21 locus deserves special attention, since it frequently becomes inactive in the early phase of urinary tract tumors. With the progression of the tumor, enhanced chromosomal instability and aneuploidy develop in the tumor cells, mostly involving chromosomes 1, 3, 7, 9, 11 and 17.[4-6]

The interphase fluorescent *in situ* hybridization (FISH) technique is the best method to detect the characteristic numerical and/or structural abnormalities of chromosomes within the cells. Fluorescent-labeled oligonucleotides (probes) are taken into the cell nucleus, where the given probe binds to its complementary cellular DNA region. The pericentromeric probes binding to the central region of the chromosomes are suitable for the detection of the number of the target chromosome, whereas the locus-specific probes provide information on the presence or absence of a given chromosomal region.

Accurate urothelial tumor diagnostics requires that the alterations of several chromosomes be studied simultaneously. Therefore, the best approach is to hybridize various probes for different pericentromeric and other chromosomal regions in a single step. This, however, demands that the features of individual probes should be suitable for the same reaction condition. This requirement will be more and more complicated to fulfill with increasing number of the probes. Consequently, developing an appropriate hybridization mix containing numerous probes is hardly achievable in routine molecular diagnostic laboratory practice. To overcome this, the Vysis company developed the UroVysion Bladder Cancer Recurrence Kit, which is currently the only FDA-approved FISH kit for the detection of urothelial carcinomas. The kit comprises a mix of probes for the detection of the 9p21 locus as well as the peri-centromeric region of chromosomes 3, 7 and 17.[4-6]

Therefore, we aimed to evaluate the utility of UroVysion test verified by the histological alterations of the urothelium.

Materials and Methods

Patients and control

Our studies were conducted using urinary bladder tissues and urine samples of 55 patients who came through cystoscopy based on preliminary clinical data (*Table 1*). These cases included 43 histologically verified urothelial carcinoma patients, and to serve as controls, 6 patients with inflammatory, 2 patients with hyperplastic and 2 with benign tumorous (papilloma) alterations. Furthermore, samples originating from 2 clinically and histologically verified tumor-free patients not showing signs of any specific histological alterations were also subjects of the study. The study protocol was in accordance with the eth-

Table 1. **FISH results of urine samples involved in the study**

Diagnosis (based on histology)	*FISH results*			*Total*
	positive	*negative*	*unevaluable*	
Malignant				
Urothelial carcinoma	34	5*	4	43
Without malignancy				
Inflammation	–	5	1	6
Hyperplasia	–	2	–	2
Papilloma	–	2	–	2
No deviations	–	2	–	2

*all stage Ta

ical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethical Committee of Semmelweis University, Budapest.

Urine preparation

According to the instructions of Vysis FISH Pretreatment Reagent Kit (Vysis, Inc., Downers Grove, IL, USA), within half an hour following the morning's first and second urinations of the studied patients, 33 ml of the urine specimens were mixed with 17 ml of Carbowax solution (2% Polyethylene glycol 1550 [SERVA Electrophoresis GmbH, Heidelberg, Germany] in 50% ethanol [Merck KGaA, Darmstadt, Germany]), which allowed for the fixed samples to be kept for 72 hours at 4°C till further processing. The samples were then sedimented by centrifugation (600 g for 10 min) and the supernatant was discarded. Cells were then washed in 10 ml PBS, followed by another centrifugation at 600 g for10 min, then the supernatant was discarded again. The precipitated cells were resuspended in a small amount of supernatant remaining in the tube, then after adding 5 ml of fresh Carnoy fixative (methanol [Fluka Chemie GmbH, Buchs, Switzerland]: glacial acetic acid [Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany] 3:1) the suspension was kept at –20°C for at least 30 min. If a sufficiently clear pellet was gained after centrifugation at 600 g for 5 min, it was dissolved in 500 µl fixative; otherwise the Carnoy fixative washing step was repeated.

Preparation of smears

In drops of 3 µl, 10 µl and 30 µl, the cell suspension was mounted onto slides which were air dried, then placed under a microscope to mark the most suitable cell density by encircling.

Pretreatment

The completely dry smears were placed into 2x SSC (sodium chloride and sodium citrate) solution and incubated at 37°C for 60 min, then washed in 0.5 mg/ml pH 1.0 pepsin solution for 15 min at 37°C, followed by washing in 1x PBS for 5 min. This step was followed by post-fixation in 1% formaldehyde for 5 min, 1x PBS for 5 min, 70-85-100% ethanol (Merck KGaA, Darmstadt, Germany) for 1 min each, and the smears were then left to dry.

FISH

According to the instructions of UroVysion Bladder Cancer Recurrence Kit (Vysis, Inc.), the DNA probe mixture was added in quantities of 3 µl onto the marked area of the slides containing the cells, and covered with coverslip hermetically closed with rubber cement. The slides were then placed on the in *situ* plate of an Eppendorf Master Cycler Gradient PCR apparatus (Eppendorf AG, Hamburg, Germany) for 2 min at 73°C in order to co-denaturate the probes and the cellular DNA. Hybridization followed at 39°C overnight (12-14 h). Next the rubber cement and cover plate were removed and the slides were placed into a 73°C hot solution of 0.4x SSC/0.3% NP-40 for 2 min, then into 2x SSC/0.1% NP-40 for 1 min at room temperature. The preparations were then air-dried in the dark at room temperature, then covered with anti-fading mounting medium containing 3 µl of DAPI-II nuclear stain. The UroVysion DNA probe mixture consisted of direct-labeled DNA probes for the peri-centromeric regions of chromosomes 3, 7 and 17 (Chromosome Enumeration Probe /CEP/ 3, CEP 7, CEP 17) as well as a 9p21 locus-specific direct-labeled DNA probe (Locus Specific Identifier /LSI/ 9p21). The CEP 3 was labeled by SpectrumRed (red), while CEP 7 had SpectrumGreen (green), CEP 17 SpectrumAqua (light blue), whereas LSI 9p21 SpectrumGold (golden yellow) labeling.

Fluorescent microscopy

Leica DM RXA motorized fluorescent microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with SpectrumRed, SpectrumGreen, SpectrumAqua, SpectrumGold single band pass filters, as well as DAPI/FITC/Texas Red triple band pass filter was used to evaluate the hybridization results. Images of detected fluorescent signals were taken at 1000-fold magnification by a Pieper FK-7512-IQ high performance monochrome CCD camera (Pieper GmbH, Schwerte, Germany) controlled by Leica CW4000 FISH software (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK).

Criteria of evaluation

Cytogenetic abnormalities fulfilling the positivity criteria of the UroVysion Bladder Cancer Recurrence Kit were determined according to instructions: the sample was considered positive if no LSI 9p21 signal was found in at least 12 cells or if at least two of CEP 3, CEP 7 and CEP 17 showed gain in at least 4 cells (=3 or more signals/cell). At least 25 abnormal cells were counted. Otherwise the analysis of the specimen was continued until one of the UroVysion test positivity criteria was met or the entire sample was analyzed. Abnormal cells were defined in our study as cells showing cytological abnormality and/or fulfilling the cytogenetic abnormality criteria of the UroVysion Bladder Cancer Recurrence Kit and/or featuring other cytogenetic alterations (9p21 incomplete deletion, multiplication of 9p21, deletion of chromosome 3, 7, or 17) detected by the UroVysion test. Criteria for cytological abnormality were

based on morphological alterations, such as enlarged, abnormal cell size as well as increased nucleus to cytoplasm ratio.

Statistical analysis

In every case, severity of the genetic alterations was defined as the percentage of cells showing cytogenetic abnormalities listed in positivity criteria of the UroVysion Bladder Cancer Recurrence Kit among abnormal cells (number of cells showing gain in at least two of the 3, 7 and 17 chromosomes and/or showing complete deletion of 9p21 locus/total enumerated abnormal cells 100). Groups were formed from the urothelial carcinoma cases based on the stage and grade determined by routine histological examinations of the tumors [Ta (9 cases)/T1 (24 cases)/T2 (10 cases); G1 (9 cases)/G2 (18 cases)/G3 (16 cases)]. Statistical differences in the severity of the genetic alterations between these groups were analyzed by Mann-Whitney test (MW test) as well as Kruskal-Wallis / Dunn's Multiple Comparison tests (K-W/DMC tests) using GraphPad Prism 2.01 statistical analysis software (GraphPad Software, Inc., San Diego, CA, USA).

Results

The cancer cell-specific chromosomal alterations detected in the urine samples by *in situ* fluorescent hybridization technique were compared with the histological findings of the transurethral resection specimens. Positivity criteria of UroVysion test were met in 34 cases; histology verified bladder cancer in all 34 patients. Negative results were obtained in 16 cases from which 5 proved to be carcinomas of superficial stage Ta, based on the later histological findings. Of these 5 cases 3 were 5 mm in size or smaller. The detailed results are shown in *Tables 1 and 2*. Altogether, based on the histological findings the specificity of the FISH test was rated as 100% and the sensitivity as 87%.

Of the 12 cases with duplicated FISH test (FISH was not only performed from the morning's second urination according to standard procedure, but from the first as well), in 3 of the 9 urothelial carcinomas both results were positive, in four cases one test was positive while the other could not be evaluated, in one case one test was positive and the other negative, whereas in one case one of the results was negative while the other could not be evaluated. Accordingly, 8 cases proved to be positive and 1 negative. Both FISH tests turned out negative in one of the inflammatory cases as well as in one of the cases devoid of any specific histological alterations, whereas one sample was negative, the other could not be evaluated in one of the papilloma cases. Thus all three cases without malignancy turned out to be negative with duplicated FISH test. There was no significant difference in the adequacy of the morning's first and second urination samples; the unevaluable FISH tests were evenly distributed among the two groups.

***Table 2.* FISH results of stage Ta (superficial) urothelial carcinomas**

Diagnosis (based on histology) (based on histology)	*FISH results*		
	positive	*negative*	*unevaluable*
Stage Ta urothelial carcinoma	3*	5**	1
Grade	*1 G1, 2 G2*	*5 G1*	*1 G2*

*All 3 tumors were larger than 5 mm or were multiplex
**Three of the 5 tumors are 5 mm in size or smaller

Of the 34 FISH-positive transitional carcinoma cases 2 stage T1 and 2 stage T2 cases displayed a complete loss of 9p21 to be the main genetic alteration (*Fig. 1*) (12%). In 30 cases (88%), including the 3 FISH positive cases of tumors with stage Ta, the gain of chromosomes 3, 7 and 17 was manifest (*Fig. 2*). Further, in certain cases (2 of 30), the multiplication of the 9p21 locus was also present (*Figs. 3 and 4*). On the contrary, in 8 cases not only 3/7/17 multiplication was observed within one and the same cell, but complete 9p21 deletion was detected as well (*Fig. 4*). Altogether 11 cases (32%) showed complete 9p21 loss.

Statistical analyses showed significant correlation between tumor progression and the rate of the cytogenetic alterations matching the positivity criteria of the UroVysion test among abnormal cells. Namely, the frequency of the cells showing cytogenetic abnormalities defined by the UroVysion test was significantly higher in T1 and T2 (invasive) bladder carcinoma cases compared to the superficial, noninvasive Ta stage ($p<0.05$ by both MW test and

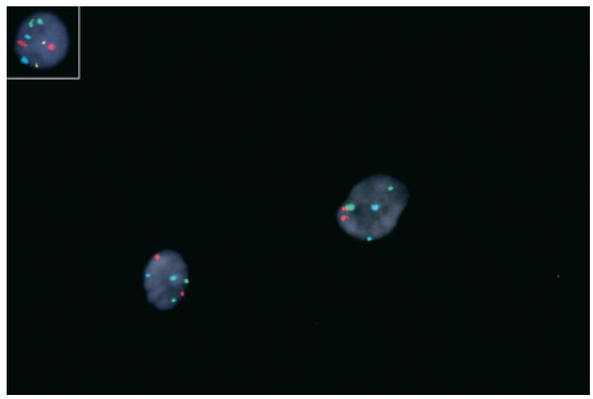

Figure 1. *9p21 deletion. The specific SpectrumGold signal (golden yellow labeling indicating the presence of locus 9p21) is missing from the cell nuclei. The left upper insert shows a normal cell as control. CEP 3: SpectrumRed (red), CEP 7: SpectrumGreen (green), CEP 17: SpectrumAqua (light blue) labeling, nuclear staining: DAPI. Original magnification: 1000x.*

K-W/DMC tests), while no significant statistical relationship was found comparing the T1 and T2 stages (*Fig. 5*). Furthermore, there was also a positive correlation between the frequency of UroVysion test-positive cells and tumor grade (*Fig. 6*): significant differences were indicated by the MW test comparing the G1 vs. G3 ($p<0.01$) as well as G1 vs. G2 ($p<0.05$) and G2 vs. G3 ($p<0.05$) cases. From this aspect, however, the more strict K-W/DMC tests found statistically significant relationship between the G1 and G3 cases only ($p<0.01$).

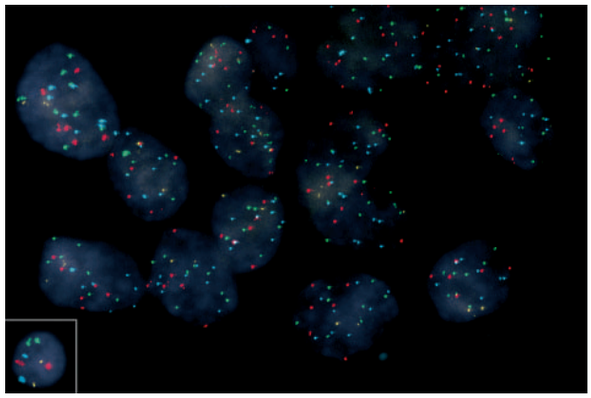

Figure 2. *Multiplication of chromosomes 3, 7 and 17, verified by more than two red, green and light blue signals per cell nucleus. The normal two copies of golden yellow signals referring to the presence of the 9p21 locus are observable in every nucleus. The lower left insert shows a normal cell as control. The differences in morphologic phenotype are well presented: the tumor cell nuclei have irregular shape and are considerably larger than normal. CEP 3: SpectrumRed (red), CEP 7: SpectrumGreen (green), CEP 17: SpectrumAqua (light blue), LSI 9p21: SpectrumGold (golden yellow) labeling, nuclear staining: DAPI. Original magnification: 1000x*

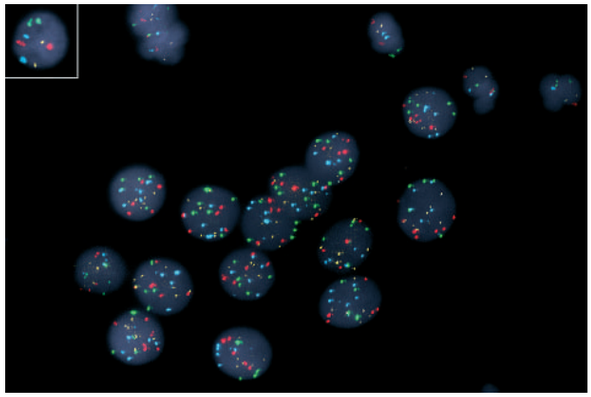

Figure 3. *3/7/17 and 9p21 multiplication, verified by more than two red, green and light blue signals and by more than the normal two copies of the golden yellow signals per cell nucleus. A normal cell can be seen in the left upper insert, as control. No phenotypic differences are observable: the tumor cell nuclei are similar to normal ones, with regular shapes and sizes. CEP 3: SpectrumRed (red), CEP 7: SpectrumGreen (green), CEP 17: SpectrumAqua (light blue), LSI 9p21: SpectrumGold (golden yellow) labeling, nuclear staining: DAPI. Original magnification: 1000x*

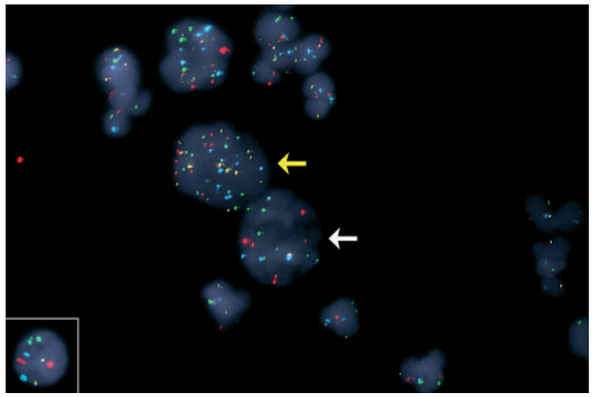

Figure 4. *3/7/17 and 9p21 multiplication and 9p21 deletion. Multiplication of chromosomes 3, 7 and 17 is shown by more than two red, green and light blue signals in the tumor cell nuclei indicated by arrows. The yellow arrow points to a nucleus in which more than the normal two copies of the golden yellow signal (referring to the 9p21 locus) are detectable. The white arrow indicates the complete deletion of 9p21 in the neighboring nucleus. The lower left insert shows a normal cell as control. Differences in cytological phenotype are represented as irregularly shaped and considerably larger than normal nuclei of tumor cells. CEP 3: SpectrumRed (red), CEP 7: SpectrumGreen (green), CEP 17: SpectrumAqua (light blue), LSI 9p21: SpectrumGold (golden yellow) labeling, nuclear staining: DAPI. Original magnification: 1000x*

Discussion

Urinary bladder cancer is a rather frequently occurring malignant disease, with 200,000 new male cases and 60,000 new female cases diagnosed annually throughout the world.[7] Urinary cytology has been used since 1945 for screening high-risk patients.[8] Despite the method's advantage of being non-invasive as well as being quite sensitive in the case of differentiated tumors, it also has disadvantages such as being examination-dependent.[8] Recent years have seen trials on several novel techniques, tumor markers and special cytological examinations, all of them aiming the detection of bladder tumors. The vast majority of data have been published on the BTA (Bard's bladder tumor antigen) test, NMP22 (nuclear matrix protein 22), TPA (tissue polypeptide antigen), fibrin degradation products, telomerase activity measurements, the microsatellite analysis and the fluorescent *in situ* hybridization technique (FISH).[9-14] As yet, not a single method has been described that could entirely replace urinary cytology and cys-

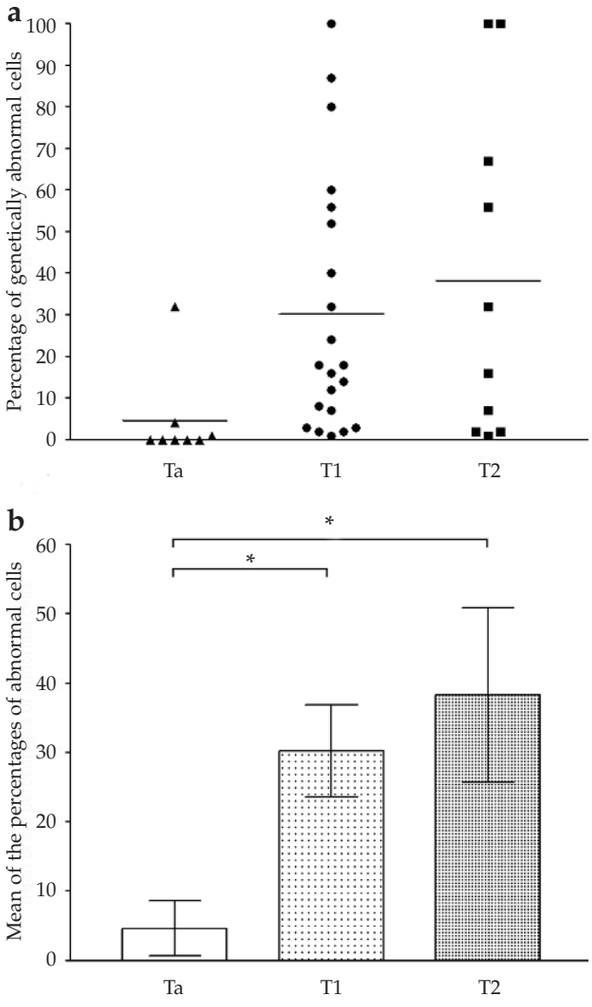

Figure. 5. *Statistical analysis of stage-related cytogenetic abnormalities. The frequency of the cells showing UroVysion test-defined cytogenetic abnormalities* ***(a)*** *among abnormal cells was significantly higher in T1 and T2 (invasive) bladder carcinoma cases compared to the superficial, noninvasive Ta stage ($p < 0.05$ by both MW test and K-W/DMC tests), while no statistically significant relationship was found comparing the T1 and T2 stages* ***(b).*** *Horizontal lines in* ***(a)*** *indicate means, while columns and bars in* ***(b)*** *represent mean ± SEM. *Statistically significant difference*

toscopy. Nonetheless, high sensitivity and specificity could be achieved with the combination of these tests.

The fluorescent *in situ* hybridization technique called UroVysion was developed in 2000 and was approved by the FDA for the detection of urothelial carcinomas in July, 2001.[4,5] Halling and colleagues tested the urine of 265 patients directly prior to cystoscopy, comparing the sensitivity and specificity of the BTA test, the hemoglobin rapid test, the telomerase test and the UroVysion technique.[5] They found the specificity (96%) and sensitivity (81%) of the UroVysion test to be the highest of the four methods. The BTA test proved to be the second best in view of sensitivity (78%), and the telomerase reaction regarding specificity (91%). Skacel and co-workers studied the urine of 120 patients using FISH and found its sensitivity to be 85%.[15] They noted that the method detected the presence of a tumor even in case of in situ carcinoma in 8 out of 9 cases, despite negative results obtained by urinary cytology.

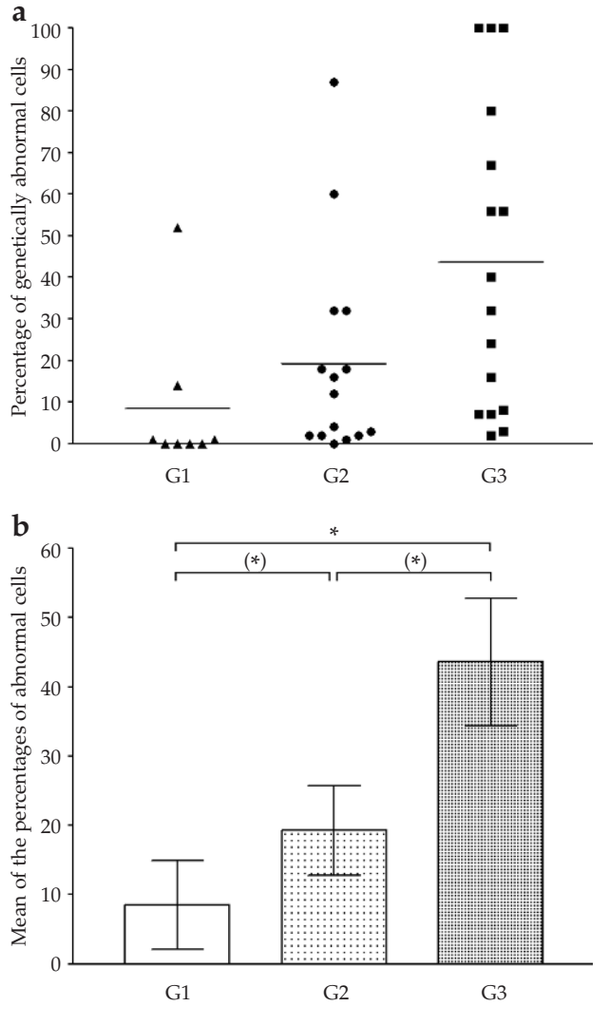

Figure 6. *Statistical analysis of grade-related cytogenetic abnormalities. The severity of the genetic alterations, described by the frequency of UroVysion test-positive cells* ***(a)*** *showed positive correlation with the histological grade* ***(b):*** *significant differences were indicated by the MW test comparing the G1 vs. G3 ($p<0.01$) as well as G1 vs. G2 ($p<0.05$) and G2 vs. G3 ($p<0.05$) cases. From this aspect, however, the more strict K-W/DMC tests found statistically significant relationship between the G1 and G3 cases only ($p<0.01$). Horizontal lines in (a) indicate means, while columns and bars in* ***(b)*** *represent mean ± SEM. *Statistically significant difference by both MW and K-W/DMC tests; (*) difference is statistically significant by the MW test only.*

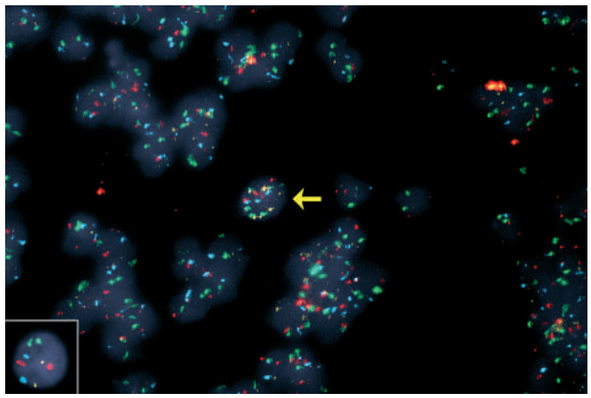

Figure 7. *The large number of granulocytes which are seen adhering to the epithelial cells makes the recognition of tumor cells extremely difficult. The present figure displays only a single tumor cell (yellow arrow) amongst many granulocytes, with the manifestation of more than the normal two copies of red, green and light blue signals representing chromosomes 3, 7 and 17, as well as the golden yellow signal referring to the 9p21 locus. The lower left insert shows a normal cell as control. CEP 3: SpectrumRed (red), CEP 7: SpectrumGreen (green), CEP 17: SpectrumAqua (light blue), LSI 9p21: SpectrumGold (golden yellows) labeling, nuclear staining: DAPI. Original magnification: 1000x*

Using the UroVysion technique, Bollmann and colleagues found close relationship between the severity of chromosomal aberrations and the progression of the disease studying 47 bladder tumor patients.[6] Our results support these findings, because the severity of the genetic alterations (described by the frequency of cells matching the cytogenetic abnormalities listed in positivity criteria of the UroVysion test among the abnormal cells) showed positive correlation with both the tumor invasiveness (stage Ta → T1, T2) and the histological grade (G1 – /G2/ → G3). Our data indicate that the rate of chromosomal abnormalities detected by the UroVysion test represents continuous changes regarding tumor grade. Invasiveness, on the other hand, is a determining step in urothelial tumor progression which correlated with a significantly higher rate of cytogenetic alterations listed in the positivity criteria of the UroVysion test.

In our own study, we found the method's specificity to be 100% and the sensitivity 87%. It should be noted that we were able to detect all the invasive bladder tumors (except the cases with unevaluable FISH result), since all the five FISH-negative urothelial carcinoma cases were of Ta stage. Ta stage solitary tumors smaller than 5 mm could not be detected by the UroVysion method. Since we worked with a higher number of cells in accordance with the diagnostic criteria, the establishment of the diagnosis was easier and more straightforward in the cases of more advanced tumor grade. On many occasions the phenotype of the tumor cells and normal cells was mostly the same (*Fig. 3*), which is indicative of how limited tumor diagnostics is, based solely on urinary cytology without the detection of any genetic deviations.

Further, the multiplication of both chromosomes 3/7/17 and the 9p21 locus was manifest in the tumor cells in 2 cases, while the simultaneous occurrence of „basic" cytogenetic alterations listed in UroVysion Bladder Cancer Recurrence Kit (9p21 complete deletion and 3/7/17 multiplication) were detected in 8 cases. These findings all refer to the complexity of the genetic background associated with tumor formation and progression. In all probability, the gain of 9p21 does not indicate an amplification specifically involving the 9p21 region, but rather indicates the multiplication of chromosome 9 (as the sign of genetic/chromosomal instability).

With the UroVysion technique the final evaluation of the samples is influenced by several factors, such as technical problems (late processing, too many or too few washings of the cells, etc.) and the characteristics of the urine sample (the chemical features of the urine, mass granulocyte infiltration caused by inflammation frequently accompanying tumors, bacteriuria, the time point of urination /the morning's first urination or the second one after high fluid intake/, etc.). Technical problems can mostly be overcome if the procedure is well organized and the specifications of the method are strictly met. It is quite frequent, however, that the sample cannot be evaluated due to the inherent characteristics of the urine. The presence of too many granulocytes and their association with epithelial cells (*Fig. 7*) can therefore make evaluation extremely difficult, as can also massive bacteriuria (*Fig. 8*). In such cases bac-

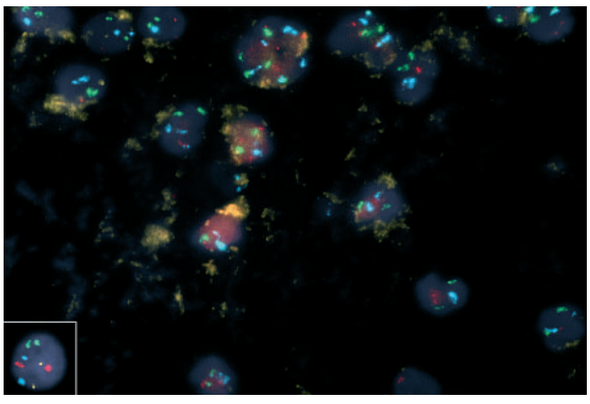

Figure 8. *Bacteriuria: Bacteria mostly fluorescing in the wavelength range of SpectrumGold (golden yellow) are adhered to the cells, making the correct detection of the 9p21 signals practically impossible. The left lower insert shows a normal cell as control. CEP 3: SpectrumRed (red), CEP 7: SpectrumGreen (green), CEP 17: SpectrumAqua (light blue), LSI 9p21: SpectrumGold (golden yellow) labeling, nuclear staining: DAPI. Original magnification: 1000x*

teria, mainly fluorescing in the wavelength range of SpectrumGold, adhere to the cells, making correct detection of the 9p21 signals practically impossible. The more concentrated first urine of the morning may contain a higher number of cells, a fact favorable in the event of few tumor cells, a large quantity of granulocytes, however, may deteriorate the quality of the sample. Furthermore, the competence and experience of the evaluating person may also be a critical factor. It is important not to evaluate the multiple signals of cells gliding onto one another as a chromosome multiplication. To avoid this, it is imperative to always carefully examine the channel of the nuclear staining (DAPI). Performing the UroVysion test on both the morning's first and, after forced drinking, the second urine samples can greatly help to eliminate the errors deriving from the different quality of the samples, and according to our experiences, can greatly decrease the number of unevaluable cases.

Both in itself and in addition to urinary cytology, the UroVysion technique produced excellent results. However, since the method is rather costly and time-consuming, it is unlikely to become widely used in routine diagnostic protocol. At present there is no other method that can be used instead of cystoscopy, but UroVysion could be an additional diagnostic tool providing help in doubtful cases.

References

1. *Droller M.J:* Bladder cancer: state-of-the-art care. Ca: Cancer J Clin 48: 269-284, 1998
2. *Oosterlinck W, Lobel B, Jakse G, Malmstrom PU, Stockle M, Sternberg C:* Guidelines on bladder cancer. Eur Urol 41: 105-112, 2002
3. *Heney NM, Ahmed S, Flanagan MJ, Frable W, Corder MP, Hafermann MD, Hawkins IR:* Superficial bladder cancer progression et recurrence. J Urol 130: 1083-1086, 1983
4. *Halling KC, King W, Sokolova IA, Meyer RG, Burkhardt HM, Halling AC, Cheville JC, Sebo TJ, Ramakumar S, Stewart CS, Pankratz S, O'Kane DJ, Seelig SA, Lieber MM, Jenkins RB:* A comparison of cytology and fluorescence in situ hybridization for the detection of bladder cancer. J Urol 164: 1768-1775, 2000
5. *Halling KC, King W, Sokolova IA, Karnes RJ, Meyer RG, Powell EL, Sebo TJ, Cheville JC, Clayton AC, Krajnik KL, Ebert TA, Nelson RE, Burkhardt HM, Ramakumar S, Stewart CS, Pankratz VS, Lieber MM, Blute ML, Zincke H, Seelig SA, Jenkins RB, O'Kane DJ:* A comparison of BTA stat, hemoglobin dipstick, telomerase and Vysis UroVysion assays for the detection of urothelial carcinoma in urine. J Urol 167: 2001-2006, 2002
6. *Bollmann M, Heller H, Bankfalvi A, Griefingholt H, Bollmann R:* Quantitative molecular urinary cytology by fluorescence in situ hybridization: a tool for tailoring surveillance of patients with superficial bladder cancer? BJU Int 95: 1219-1225, 2005
7. *Parkin DM, Pisani P, Ferlay J:* Estimates of the worldwide incidence of 25 major cancers in 1990. Int J Cancer 80: 827-841, 1999
8. *Glas AS, Roos D, Deutekom M, Zwinderman AH, Bossuyt PM, Kurth KH:* Tumor markers in the diagnosis of primary bladder cancer. A systematic review. J Urol 169: 1975-1982, 2003
9. *Grossman HB:* New methods for detection of bladder cancer. Semin Urol Oncol 16: 17-22, 1998
10. *Slaton JW, Dinney CP, Veltri RW, Miller CM, Liebert M, O'Dowd GJ, Grossman HB:* Deoxyribonucleic acid ploidy enhances the cytological prediction of recurrent transitional cell carcinoma of the bladder. J Urol 158: 806-811, 1997
11. *van Poppel H, Billen J, Goethuys H, Elgamal AA, Gerits M, Mortelmans L, Blanckaert N, Baert L:* Serum tissue polypeptide antigen (TPA) as a tumor marker for bladder cancer. Anticancer Res 16: 2205-2208, 1996
12. *Zhang FF, Arber DA, Wilson TG, Kawachi MH, Slovak ML:* Toward the validation of aneusomy detection by fluorescence in situ hybridization in bladder cancer: comparative analysis with cytology, cytogenetics, and clinical features predicts recurrence and defines clinical testing limitations. Clin Cancer Res 3: 2317-2328, 1997
13. *Mao L, Schoenberg MP, Scicchitano M, Erozan YS, Merlo A, Schwab D, Sidransky D:* Molecular detection of primary bladder cancer by microsatellite analysis. Science 271: 659-662, 1996
14. *Steiner G, Schoenberg MP, Linn JF, Mao L, Sidransky D:* Detection of bladder cancer recurrence by microsatellite analysis of urine. Nature Med 3: 621-624, 1997
15. *Skacel M, Fahmy M, Brainard JA, Pettay JD, Biscotti CV, Liou LS, Procop GW, Jones JS, Ulchaker J, Zippe CD, Tubbs RR:* Multitarget fluorescence in situ hybridization assay detects transitional cell carcinoma in the majority of patients with bladder cancer and atypical or negative urine cytology. J Urol 169: 2101-2105, 2003