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RESEARCH

The Value of the Preoperative FISH Test in Unscreened Bladder Cancer Patients with TUR Indications

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Abstract Patients with bladder cancer are still requiring close follow up with frequent cystoscopies. This study aims to assess the FISH analysis, as a procedure capable of highlighting the hidden features of a tumor and helping to individualize treatment tactics. The bladder washings of 50 primary bladder cancer patients were taken prior to TURB and analyzed with the commercial FISH assay UroVysion®. All patients were divided into groups according to the maximum stage and grade of the tumor. The sensitivity of the method was 81.5 %, 91.7 % and 100 % for the Ta, T1 and T2 stage groups, respectively. For the G1, G2 and G3 groups the sensitivity was 70 %, 100 % and 100 %, respectively. In addition, the rate of detecting genetically abnormal cells was significantly higher in the T2 stage compared to the Ta and combined Ta+T1 groups, as well as in the G3 group compared to the G1 and G2 groups. The mean signal number from each chromosome insignificantly increased with the stage and grade of the tumor. The detection of <40 % genetically abnormal cells predicted the absence of muscle invasion and a G3 tumor with more then 90 % reliability. The FISH method is highly sensitive in early bladder cancer detection and is able to predict the morphological character of a tumor even before surgery.

Keywords Bladder cancer · Urothelial carcinoma · FISH · Prediction · Bladder washings

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Introduction

Morbidity due to bladder cancer is steadily increasing. In Russia, according to all the available information this disease in the structure of oncopathology takes 4.6 % in men (5th place) and 1 % in women. In 2007 approximately 13,000 new cases of bladder cancer were registered in Russia [1]. In 70–75 % of cases the cancer is diagnosed at an early stage without invasion of a muscular layer [2, 3]. This was the motivation for the wide application of spare organ surgery and specifically transurethral resection. However, the fact that urothelial cancer cells are extremely variable in terms of biological characteristics, there is a high rate of relapses and a significant risk of progression, means doctors are more likely to follow-up patients and cystoscopies are performed frequently. This diagnostic procedure has both advantages and shortcomings, including invasiveness and high costs. Another widespread method used to diagnose bladder cancer is cytologic urinalysis but this is not sufficiently sensitivity in the early stages of the disease [4]. Therefore, various markers that are potentially capable of predicting the course of the disease and effectively

diagnosing its early relapses are intensively investigated. One such method is the FISH diagnosis which is based on the commercial set UroVysion® (Abbott Laboratories, the USA).

In early studies on this topic it was found that homozygous deletion of 9p21 locus is one of the most common genetic alterations in early stages of urothelial cancer [5, 6]. Additionally, cytogenetic studies revealed common changes in chromosomes 1, 7, 9, 11 and 17. In a study by Sokolova et al. it was shown that the highest sensitivity in detecting bladder cancer was achieved by complex detection of 9p21 locus loss and aneuploidy by chromosomes 3, 7 and 17 [7]. The fluorescent labels to these chromosomes were included later in the commercial assay UroVysion®.

Discussions are currently being held about whether this method is capable of predicting the course of the disease. This study aims to assess the FISH diagnosis as a method that is capable of potentially highlighting the latent features of the tumor and helping with determining a choice of patient treatment tactics.

Patients and Methods

The 50 primary bladder cancer patients treated at the Kazan Cancer Center in 2009 and 2010 were considered in the prospective research. The primary inclusion criteria for the study were cystoscopic tumor findings that could be radically removed with TUR. All of the patients were informed about the forthcoming study and have given their voluntary consent to participate in it. All of the patients have passed standard complex ambulatory work up to exclude regional and remote metastases. In all cases exophitic tumors without visible signs of muscle invasion were detected during diagnostic cystoscopy. Transurethral resection was performed on all patients. Bladder washings were taken immediately before the operation on the operating table using a sterile physiological solution (100–150 ml). The surgical material was studied by at least two certified oncopathologists and the results for each case included all the information on the type of tumor growth, the depth of germination and the degree of tumoral cells anaplasia (grade) according to the WHO of 1973.

FISH Assay Bladder washings were centrifuged no later than 1 h after they were conducted and following this the centrifuged material was selected and the supernatant fluid was removed. Then cellular sediment was exposed in preheated to 37 °C 0.56 % KCl water solution within of 10 min at a room temperature. Further, the received solution was centrifuged again up to a volume of two containers (each 1.5 ml). Subsequently, the supernatant fluid was replaced stage-by-stage by a preservative prepared ex tempore (methanol and acetic acid at a ratio of 3:1). The centrifugation and preservative replacement were consecutively performed 3 times in each case. Next we immediately prepared smears from the received material by Cytospin® centrifuge (2–6 glasses for each case), selecting satisfactory cellular density. The received material (smears and suspension of preserved cells) was stored at−20 °C. Later, the received smears were specially treated according to the UroVysion® assay kit instructions, with a few changes. Thus, the length of time the smears spent in the protease solution increased up to 30 min and the temperature of the formamid solution increased for denaturation from 73 °C to 75 °C. Then we performed hybridization from the DNA test which contained 4 probes labeled by fluorochromes. The test includes probes that complemented the centromere sites of the 3rd chromosome (red label SpectrumRed), the 7th chromosome (green label SpectrumGreen) and the 17th chromosome (blue label SpectrumAqua) and also probe to the 9p21 locus on the 9th chromosome (yellow label SpectrumGold). After the slides had been in the hybridizer for 16 h at 37 °C, they were washed and the DAPI stain was added. Then we performed fluorescent microscopy on the received smears using a Leica DM 5000B microscope with the following preset light filters: DAPI single, Aqua single, Yellow single and Red/Green dual.

Each preparation was studied to detect 100 urothelial cells with a visually abnormal nucleus which was characterized by the increased sizes, rough contours and non-uniform painting by DAPI. The quantity of signals for each label in an abnormal nucleus was fixed. The cells that had overlapping nuclei were not counted. The signals situated in contact to each other were counted as one signal (Figs. 1, 2 and 3).

Some additional parameters of the FISH analysis estimated in each case are given in detail in Table 1.

The formal conclusion about the presence of cancer was performed by criteria recommended by the manufacturer.

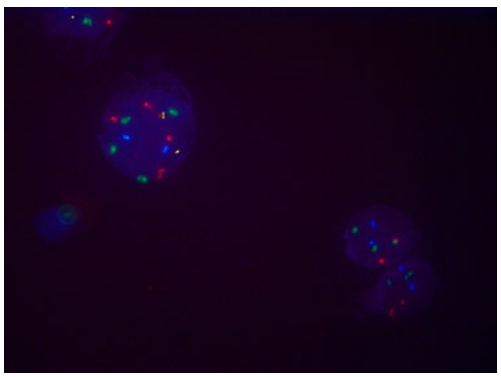

Fig. 1 Chromosome 3 and 7 hyperploidy (5 red and 4 green signals, respectively) in the cell on the left and homozygous deletion of 9p21 locus (no gold signals) in two tumor cells in the right bottom corner. FISH method. x1000

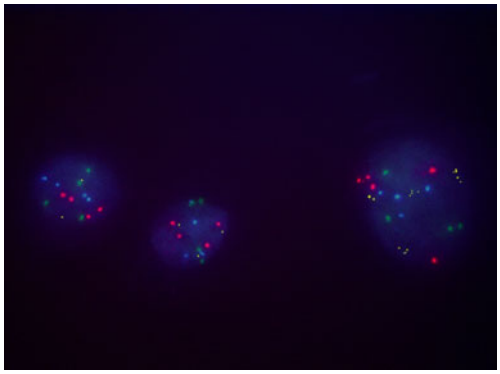

Fig. 2 Tetraploidy by 3, 7 and 17 chromosome in all three tumor cells. Normal (2) number of Gold signals (locus 9p21), 3 and 4 signals from 9p21 locus in 2nd and 3rd cells to the left, respectively. FISH method. x1000

Thus, detection of 4 or more cells with hyperploidy on 2 or more centromere labels or 12 or more cells with homozygous deletion of the 9p21 locus on the 9th chromosome was considered to be a positive result. The reassessment of formal FISH test results was done using modified criteria: up to 5 cells with tetrasomy by all 4 labels (chromosomes 3, 7, 17 and 9p21 locus) were excluded from the number of cells with chromosomal abnormalities.

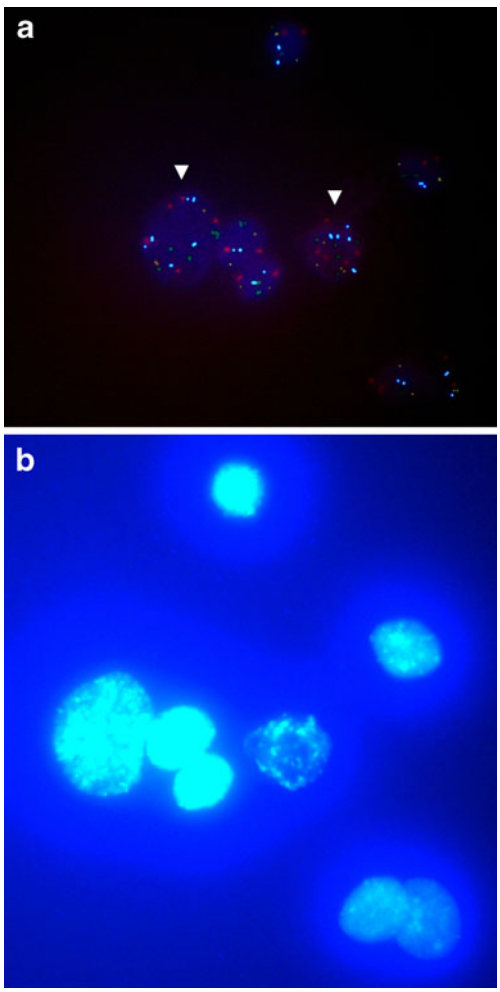

Fig. 3 **a** Abnormal number of signals in two large tumor cells (pointed by *arrow*). In left cell: 5 red, 4 green, 6 aqua and 2 gold signals; in right cell: 5 red, 5 green, 5 aqua and 4 gold signals. Normal number of signals in small cells lying in between (inner control). **b** The same field of view in DAPI filter. FISH method. x1000

The computer based program Statistica® (StatSoft Inc., Tulsa, OK, USA) was used for statistic processing. Fisher's exact criterion was applied to estimate the significance value of the sensitivity difference. The student's t-criterion was used for pair quantitative comparisons and one-factorial dispersive analysis and Bonferroni's comparison was used for multiple quantitative comparisons. For nonparametric values comparison the Chi-squared test was used. The boundary significance value has been established at $p=0.05$.

Results

In all patients transitional cell carcinoma has been diagnosed: stage Ta, stage T1 and stage T2a in 27, 12 and 11 patients, respectively. According to the grade the results were as follows: the G1, G2 and G3 tumors were diagnosed in 20, 15 and 15 patients, respectively. No one case of cancer in situ has been revealed. Patients in all stage groups Ta, T1 and T2 differed insignificantly on the grade.

The slide selection with optimal cell density for FISH analysis was done in all cases before the hybridization. During the fluorescent microscopy a sufficient number of preserved cells (more than 100 per slide) with tumor appearing nucleus was available for analysis in all cases.

Sensitivity of the Formalized FISH Test

The sensitivity of the FISH test was 88 % (44/50) in general. All patients were divided into groups according to the maximal stage and maximal grade of the tumor. The results of the sensitivity calculation for each of the groups are shown in Table 2.

As shown, the sensitivity of the FISH assay does not vary significantly in groups with tumors Ta, T1 and T2a by paired comparison. The sensitivity in the combined Ta+T1 group also did not differ significantly compared to the T2a group.

There was a statistically significant difference in sensitivity in the G1 group compared to the G2 and G3 groups ($p=0.027$).

After the formal FISH test criteria modification there was only 1 patient whose formal result was changed from positive to negative. In total, the presence of tetrasomic cells in bladder washing samples was only found in 9 out of 50 patients. The minimum number of tetrasomic cells was 1 and the maximum was 6 cells.

Table 1 Additional parameters of the FISH analysis results estimation

Description of parameter	Name of parameter
The total number of cells with at least one chromosome anomaly on 100 tumor cells	The total number of cells
Number of cells with aneuploidy of the 17th chromosome on 100 tumor cells	Aq cells
Number of cells with aneuploidy of the 7th chromosome on 100 tumor cells	Gr cells
Number of cells with aneuploidy of the 3rd chromosome on 100 tumor cells	Rd cells
Number of cells with the 9p21 locus alteration on 100 tumor cells	Gl cells
Average of signals from the 17th chromosome in one tumor cell	Aq signals
Average of signals from the 7th chromosome in one tumor cell	Gr signals
Average of signals from the 3rd chromosome in one tumor cell	Rd signals
Average of signals from the 9p21 locus in one tumor cell	Gl signals
Average of signals from the 17th chromosome in one tumor cell	Aq signals

Distribution of Genetic Anomalies Depending on the Stage of the Disease

The general number of cells with genetic changes by at least one signal, as well as with alterations by each signal separately, rose with the increasing of the tumor stage. The mean number of cells with at least one signal aberration was: 37 for the Ta tumors, 61 for the T1 tumors and 75 for the T2a tumors. The number of cells with changes in each chromosome was lower than expected: about 20, 40 and 50 cells for the Ta, T1 and T2a groups, respectively. This trend was demonstrated better by the T2 stage group, where the difference in the number of abnormal cells on at least one signal and on each signal separately was statistically significant compared to the Ta and Ta+T1 patient groups.

The results of the assessment of genetic aberrations depending on the stage of the disease are shown in Table 3.

The number of cells with each chromosome alterations rose with increasing of the tumor stage. But these numbers were very close within single group of patients, not showing the prevalence of changes in any single chromosome (Fig. 4a). The mean number of signals in each chromosome in one tumor cell also rose as the tumor stage increased. For chromosomes 3, 7 and 17 the mean number of signals was: approximately 3 for Ta group, about 3.5 for T1 and around 3.7 for T2 stage patients. But for the locus 9p21 the mean number of signals in each tumor cell was below 2 for Ta and T1 tumors, and about 2.25 in the T2a stage group (Fig. 4b).

Table 2 Sensitivity of the FISH diagnostics in patients with different stages and grades of bladder cancer

Group	Number of patients	Number of FISH«+»results	Sensitivity (%)
Ta	27	22 (21[a])	81,5 (77,8[a])
T1	12	11	91,7
T2a	11	11	100
Ta+T1	39	33	84,6
G1	20	14 (13[a])	70* (65[a])
G2	15	15	100
G3	15	15	100

*Significant difference $p<0.05$

[a] Sensitivity after criteria modification (exclusion of up to 5 tetrasomic cells)

Distribution of Genetic Anomalies Depending on the Grade of the Tumor

There was a similar tendency in different grade groups of patients. The general number of genetically abnormal cells, as well as the number of cells with aberrations on each of the chromosomes is rising with increasing of tumor grade. The results of the assessment of genetic alterations depending on the grade of the tumor are shown in detail in Table 3.

There was a nonlinear rising of the number of cells with chromosomes 3, 7, and 17 aneuploidy, as well as the general number of genetically abnormal cells by increasing the grade from G2 to G3. Statistically significant differences were observed by pair comparison of the G1/G3 and G2/G3 groups. For locus 9p21 only the G1 and G3 groups differed significantly (Fig. 5a).

The mean number of signals from chromosomes 3, 7 and 17 rose more considerably as the grade increased from G1 to G2, which is shown in Fig. 5b. But for chromosomes 7 and 17 only the significant differences were observed by pair comparison of groups G1/G3 and G1/G2 and G1/G3, respectively. The analysis of locus 9p21 changes showed a similar distribution in G1 and G2 groups where the mean number of signals was below 2. Although it wasn't statistically significant, this number was 2.5 in the G3 tumor group.

In our study we faced the well-known heterogeneity of chromosomal number changes within a single case. To represent this finding we compared the variation of the signal number from each label for each different stage and

Table 3 Distribution of FISH results subject to the stage and grade of the tumor

Parameter	Stage groups	Mean±st. error	Significance ^	Grade groups	Mean±st. error	Significance ^
The Total of cells	Ta	37,29±4,77	T2-p<0,01	G1	32,60±4,84	G1,G2-p<0,01
	T1	60,73±10,47		G2	52,00±7,55	
	T2	74,91±7,36	Ta+T1-p<0,01	G3	78,67±7,60	
	Ta+T1	45,05±4,74				
Aq Cells	Ta	19,00±3,84	T1,T2-p<0,01	G1	14,00±3,43	G1,G2-p<0,01
	T1	45,45±10,25		G2	27,47±4,94	
	T2	53,09±9,50	Ta+T1-p<0,01	G3	66,67±8,13	
	Ta+T1	27,16±4,42				
Gr Cells	Ta	19,17±4,62	T2-p<0,01	G1	16,24±4,21	G1,G2-p<0,01
	T1	36,00±11,66		G2	24,92±6,39	
	T2	54,40±10,79	Ta+T1-p<0,01	G3	64,80±11,57	
	Ta+T1	24,50±4,80				
Rd Cells	Ta	18,71±4,23	T2-p<0,01	G1	13,60±3,66	G1,G2-p<0,01
	T1	40,36±12,13		G2	25,07±6,26	
	T2	55,27±9,96	Ta+T1-p<0,01	G3	67,33±9,04	
	Ta+T1	25,47±4,83				
GL Cells	Ta	21,71±4,14	T2-p<0,01	G1	18,40±4,25	G1-p<0,01
	T1	39,27±9,39		G2	32,00±6,18	
	T2	49,82±7,75	Ta+T1-p<0,01	G3	50,00±8,69	
	Ta+T1	27,37±4,16				
Aq Signals	Ta	2,96±0,19	p=0,2	G1	2,74±0,22	G2,G3-p<0,05
	T1	3,34±0,28		G2	3,35±0,15	
	T2	3,41±0,22		G3	3,58±0,20	
	Ta+T1	3,07±0,16				
Gr Signals	Ta	3,16±0,25	p=0,32	G1	3,02±0,25	G3-p<0,05
	T1	3,33±0,17		G2	3,54±0,20	
	T2	3,63±0,16		G3	3,78±0,11	
	Ta+T1	3,21±0,19				
Rd Signals	Ta	3,32±0,16	p=0,112	G1	3,21±0,22	p=0,14
	T1	3,75±0,23		G2	3,62±0,16	
	T2	3,73±0,13		G3	3,82±0,15	
	Ta+T1	3,45±0,13				
Gl Signals	Ta	1,53±0,31	p=0,396	G1	1,54±0,36	p=0,183
	T1	1,74±0,45		G2	1,30±0,31	
	T2	2,25±0,48		G3	2,49±0,52	
	Ta+T1	1,59±0,25				

^In the "Significance" column there is the result of the dispersion analysis or, in case of significant differences, the group is compared with and p value shown for pair comparison

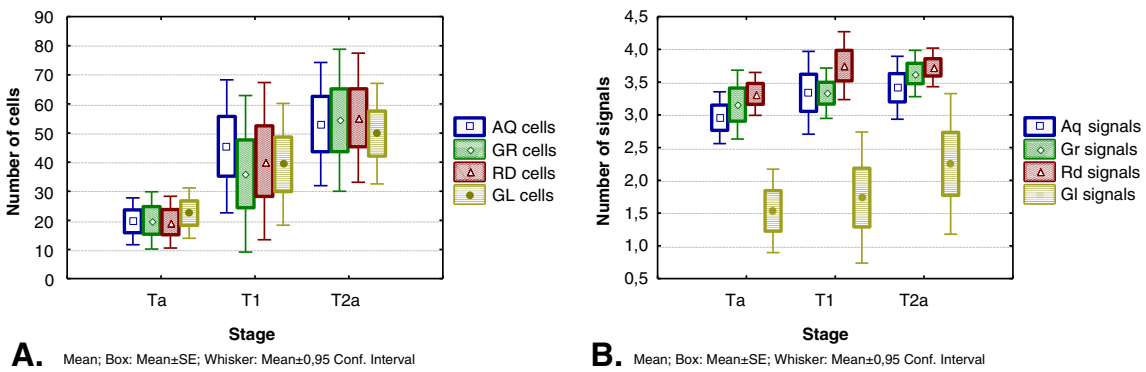

Fig. 4 The distribution of additional parameters of the FISH test subject to the tumor stage. **a** the rate of the genetically abnormal cells; **b** the mean number of signals from each chromosome

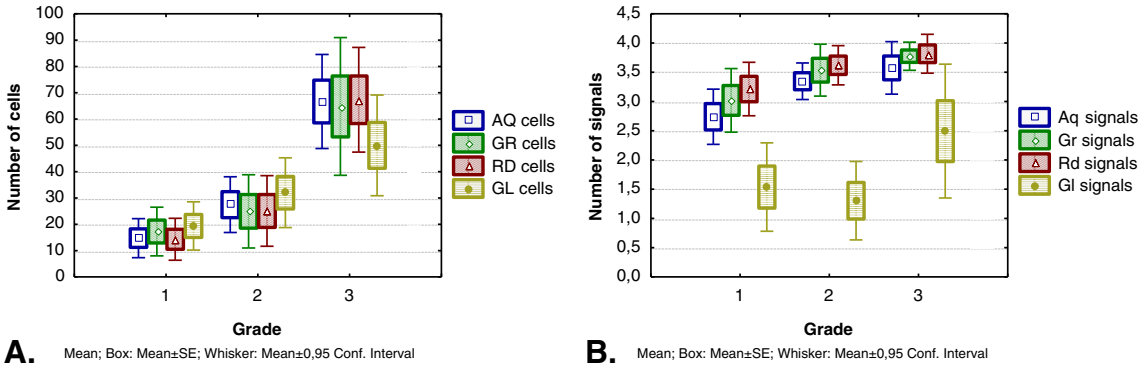

Fig. 5 The distribution of additional parameters of the FISH test subject to the tumor grade. **a** the rate of the genetically abnormal cells; **b** the mean number of signals from each chromosome

grade patient subgroups. For this reason we used the coefficient of variation (CV). To make the distinctions more visible the cells with disomy (2 copies) for a single label were excluded during CV calculation. The results of this comparison are shown in Table 4.

Considering the different types of chromosomal anomalies, we observed both anomaly types (loss and gain of copy number) at the same time in 16 cases of centromeric labels (chromosomes 3, 7 and 17). The loss of 9p21 locus (hetero- and homozygous deletion) was found in 29 cases. Although there were simultaneously detected cells in only 12 cases with a loss and gain of 9p21 locus number, the number of Gold signals was much more variable in all patients groups, compared to centromeric labels. There was no difference in any chromosome loss frequency among patients from all stage and grade groups, nor were any significant differences found in all DNA labels or the CV level between different patients' stage and grade groups.

Discussion

In the given study the FISH test UroVysion® has shown a slightly higher sensitivity (88 %), compared (on average) to other authors' results (56–100 %) [4, 8-13]. Although the univocal rising of the sensitivity of the test was shown as the stage of the tumor increased, these differences were not statistically significant. The increasing of the grade showed a more significant rising of the sensitivity of the test. In general, the assessment of the formalized FISH test showed high sensitivity in even the earliest forms of diagnosis (Ta and G1) of bladder cancer.

In other studies the tendency of the high grade tumor cells to gain chromosomes 3, 7 and 17 [14] was shown. Using additional characteristics of the FISH test we found a significant difference in the chromosomal aberration rate in the different stage and grade groups of the patients. We noticed a stepwise rising of the rate of anomalies according to the change in stage from the formerly called superficial (Ta and T1) to muscle-invasive cancer (T2), as well as from G1 and G2 tumors to high grade G3 tumors.

The results of this study did not show any significant anomaly prevalence by any of the available signals in tumor cells from different clinical and morphological groups. But as noted earlier, differences in locus 9p21 aneuploidy characteristics can indicate a change in the pattern from a predominant loss of this locus in Ta, T1 and G1, and G2 tumors to mostly increasing of this locus number in T2 and G3 tumors. Our findings correlate well with the results of other authors [15], who

Table 4 The coefficient of variation (CV) of signal number (Aqua, Green, Red and Gold) against the patient's stage and grade group

Group	Aq signal variation, CV (M±m)	Gr signal variation, CV (M±m)	Rd signal variation, CV (M±m)	Gl signal variation, CV (M±m)
Ta	0,3238±0,0525	0,2768±0,0496	0,2898±0,0374	1,0654±0,1948
T1	0,3168±0,0580	0,2364±0,0630	0,2351±0,0768	0,9544±0,4787
T2	0,3110±0,0526	0,2840±0,0450	0,2493±0,0538	0,7455±0,3303
G1	0,3172±0,0976	0,3174±0,1065	0,3387±0,0715	0,7205±0,2318
G2	0,3167±0,0470	0,2800±0,0417	0,2507±0,0510	0,8831±0,1942
G3	0,3235±0,0514	0,2351±0,0378	0,2463±0,0394	1,1861±0,3834

have revealed statistically significant differences. This makes it possible to assume that there is a low risk of progression of the tumors with a predominant loss of 9p21 locus by FISH analysis. On the other hand, in some studies it has been shown that the loss of 9p21 locus correlates with the early recurrence of tumors in the first year after resection [11, 16].

The UroVysion® diagnostic kit was designed for the primary diagnosis of bladder cancer and the labels for chromosomes 3, 7, 17 and 9p21 were included as the most frequent and typical sites of aberration. But the biological sense of genes on these chromosomes is different. Chromosomes 7 and 17q carry oncogenes EGF-R and c-erb-B2 (Her2), respectively, as well as the tumor suppressor gene Tp53 on chromosome 17p. Chromosomes 3 and 9p21 carry mainly many tumor suppressor genes: VHL, RASSF1 FHIT (chromosome 3) and CDKN2A/CDKN2B (locus 9p21). Knowing this we could expect to see a more frequent loss of chromosome 3 with tumor stage and grade progression at least. Also, earlier studies showed the frequent loss of chromosome 17p in high grade urocarcinomas [17]. Using only centromeric labels for chromosomes 3, 7 and 17, we have not found any differences in the loss frequency of these chromosomes subject to tumor stage and grade.

As mentioned above, in the majority of cases the morphological analysis of surgical biopsies after TUR shows non-muscle-invasive cancer. But treatment tactics, recurrence and progression prognosis could greatly differ, even inside this group of patients [18]. Besides, there is a high "understaging" risk in a morphological assay of classic TUR biopsies. According to some authors this risk can reach 30 % [19-22]. This fact has made popular second look TUR, which can alter the management in up to a third of cases [23].

This study lacks data regarding benign bladder neoplasm. Although urothelial papilloma is a relatively frequent finding in young patients (<20 years old), it represents less than 3 % of all urothelial tumors of the bladder in the general population [24, 25]. In practice any bladder neoplasm should be removed. EAU guidelines defend the possibility to omit the diagnostic biopsy in case the bladder neoplasm has been identified in earlier imaging studies and the patient undergoes the TUR [18]. Even if the cystoscopy was performed, the cold knife biopsy sampling could not fully represent the whole tumor, nor does it have sufficient information regarding the tumor extension into the bladder wall. Considering the importance of invasive tumor detection we take a few cells with any chromosome anomaly as a threshold value in superficial and muscle-invasive tumors discrimination (Ta+T1 and T2), as well as for high grade tumors detection (G1+G2 and G3). Achievement of the threshold number of cells was considered to be a positive result. Positive predictive value (PPV) and negative predictive value (NPV) were calculated for each of the threshold numbers (Table 5).

As shown in the table the 40 % threshold has the highest NPV for both of the factors (Stage and Grade). In practice it could make it possible to rule out with help of FISH test the presence of muscle-invasive tumors and G3 tumors in patients with less than 40 % tumor cells with the aneuploidy by at least one chromosome. Taking into account the fact that bladder washing sampling is possible during the first diagnostic cystoscopy, this gives valuable information about the treatment method selection.

Conclusion

The FISH analysis of bladder washing can accurately predict the clinicopathologic features of the tumor prior to surgery, if this is done during the first diagnostic cystoscopy. This could indicate a more differential use of early intraoperative intravesical chemotherapy, second look TUR and adjuvant intravesical therapy. In addition, the FISH test can detect T2 tumors cystoscopically indistinguishable from superficial ones, which can make it possible to select a more aggressive treatment approach for some patients.

Table 5 Predictive value of the number of cells with at least one chromosome abnormality (in 100 tumor cells)

Feature of the tumor	Threshold of the total of cells	PPV, %	NPV, %	Significance, *p*
Muscle invasion of tumor	60	38	89,7	0,036
	50	39	92,6	0,014
	40	40	96	0,0041
High grade of tumor (G3)	60	50	85,7	0,025
	50	45,5	88,5	0,011
	40	45,8	91,7	0,0078

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