Long-term Follow-up of Intermediate-risk Non-muscle Invasive Bladder Cancer Sub-classified by Multi-coloured FISH

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Abstract. *Aim: To examine the long-term follow-up of patients with that previously underwent risk stratification based on multicolour FISH testing. Patients and Methods: On 81 patients with intermediate-risk urothelial carcinoma, a multicolour-FISH was performed. Patients were sub-divided into low- and high-risk groups based on chromosomal patterns. Univariate analysis, using Mantel-Cox log-rank test for disease-free, progression-free survival and overall survival, was employed to determine the prognostic significance of FISH analysis. Survival times were calculated according to the Kaplan-Meier product-limit method and multivariate analysis using Cox proportional hazards regression model. Results: The univariate Mantel-Cox log-rank test showed significant differences between the low-risk and the high-risk group for disease-free survival (p=0.005) and overall survival (p=0.038), but not for progression-free survival (p=0.129). Conclusion: Our long-term follow-up data appear to be able to divide tumors into low and high risk groups for recurrence based on molecular/genetic changes observed with FISH.*

At the time of diagnosis the majority of urothelial cancer (UC) cases are non-muscle invasive. Approximately 40% are papillary pTa UC, 30% pT1 UC and 2-5% are carcinoma *in situ* (Cis) (1). Traditionally, these differing stages are grouped together under the heading "superficial bladder cancer" (2) as they can be removed by transurethral resection (TUR). This is however, not a homogenous entity as grade (high, intermediate and low) and different stages (pTa, pT1 and Cis) play a significant role in recurrence and progression rates. This is evident as progression occurs in 19% of patients with Ta tumours in comparison to 34% with pT1 bladder carcinoma (3).

The oncogenic potential of bladder cancer has been explored on a molecular and genetic basis. Oncogenesis appears to occur along different pathways (4) with different oncological and clinical potentials. Loss of heterozygosity of chromosome 9p and 9q has been shown to be a crucial event in the transformation from normal urothelium to papillary urothelial carcinoma while p53 is primarily involved in the development of carcinoma *in situ* (4, 5). It has been suggested that the loss of either one or both alleles of the p16 gene in human papillary urothelial cancer plays a major role in early carcinogenesis (6, 7).

Several large groups of investigators (8-10) have identified clinical, but not molecular or genetic prognostic factors that allow for the identification of three different groups with regard to risk for progression. These include the following: a. Low-risk tumors: single, pTa, G1, <3 cm diameter; b. High-risk tumors: pT1, G3, multifocal or highly recurrent, Cis; c. Intermediate-risk tumors: all other tumours.

The standard therapy for pTa, pT1 tumours is the complete macroscopic removal by transurethral resection (TUR) including sampling of the underlying muscle. The therapy for low-risk tumors is completed with one single chemotherapeutic instillation immediately after the TUR. In the high-risk group, BCG or chemotherapy instillation protocol is recommended and a maintenance therapy is necessary even if the optimal timing and schedule of

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instillation has not been determined (8). No consensus exists regarding the optimal therapy in the intermediate-risk group and less regarding the follow-up necessary for these lesions.

A first attempt to discriminate between high-risk and low-risk disease was done by the introduction of the 2004 WHO grading system which is supposed to sub-divide the critical G2 (WHO 2004). Hence, in a previous study, we tried to address this problem by performing a biological characterization of the tumors of the intermediate-risk group using multicolour-FISH. Using this technique it seemed possible to assess the biological behaviour of urothelial carcinomas more accurately. We showed that patients with a high-risk chromosomal pattern have a significantly shorter disease-free survival time and higher progression rate compared to patients with a low-risk pattern (11).

The current study examines the long-term (10 years) follow-up of these intermediate-risk patients that previously underwent risk stratification based on multicolour FISH testing.

Patients and Methods

Between 2002 and 2003, 81 consecutive patients (mean age=69.9 years, range=41-94, median=71, SD 11.2) undergoing follow-up after transurethral resection (TUR) of intermediate-risk urothelial cancer of first manifestation (22 pTaG1 multifocal or >3 cm diameter, 53 pTaG2, 6 pT1G2) were prospectively studied. Each patient received 80 mg of farmarubicin within 6 h after resection and a full-course of 7 instillations of 80 mg farmarubicin starting 21 days after resection. Patients with low risk (pTa, G1, <3 cm) and high risk (pT1, G3, multifocal or highly recurrent, or CIS) non-muscle invasive bladder cancer (NMIBC) were excluded. The patients were followed-up for a mean observation period of 79.0 months (range=6-130 months, median=95, SD 40.7) until January 2013. On all patients, a multicolour-FISH was performed on liquid-based voided urinary cytology using the UroVysion® Bladder Cancer Kit (UroVysion Kit) (Abbott Molecular, Des Plaines, IL, USA) at the first follow-up after classification. During follow-up, any cystoscopically-suspicious lesion was biopsied or removed transurethrally. Histopathological classification was performed according to the UICC criteria (12). Any new lesion after primary TUR-B of same grade or stage was defined as recurrence, any increase of grade or/and stage was scored as progression. Histopathological diagnoses are given in Table I. All histological results have been re-reviewed by a pathologist.

Cytological evaluation was then performed by using the Papanicolaou staining procedure. Diagnostic results were categorized as previously described by Koss *et al*. (13). Specimens that were negative for malignancy or had mild atypia were categorized as negative. Specimens considered as suspicious were re- reviewed for this paper and only specimens re-categorized as positive were included. The cytological diagnosis was determined by an experienced cytologist, and all positive results were confirmed by an experienced pathologist

Multicolour FISH. After pre-treatment with 2× sodium saline citrate (SSC) and 0.5 mg/ml pepsin at 37˚C and 0.01 N hydrochloric acid in a waterbath, 1% formaldehyde and phosphate buffered saline at room temperature, the slides were dehydrated in 70%, 80% and 100% ethanol. After drying the slides, the probe mix (Abbott Molecular, Des Plaines, IL, USA) containing a locus-specific probe for the locus 9p21 (p16) and three α-satellite-bound centromere-specific probes for chromosomes 3, 7 and 17 was placed on the target. Codenaturation (5 min at 73˚C) and hybridisation at 37˚C were carried out overnight in the HYBrite oven (Abbott Molecular, Des Plaines, IL, USA). The procedure was followed by a post-wash using 0.4× SSC and 0.3% Nonidet (NP40) and 2× SSC and 0.1% NP40. Diamidinophenylindole II was used as a counterstain.

Slides were scored for hybridisation signals on a cell-by-cell basis, using an Olympus Provis BX61 (Olympus Italia, Milan, Italy) with a filter set including diamidinophenylindole single band pass (counterstain), aqua single bandpass (chromosome 17), yellow single bandpass (9p21 locus) and a red–green double bandpass (chromosomes 3 and 7). Enumeration and evaluation of the FISH signals was carried out on target cells that appeared abnormal morphologically, according to Bubendorf *et al*. (6), and the cut-off level was set at >4 aneusomic cells. According to Pycha *et al*. (14), patients in this intermediate category were then sub-divided into low- and high-risk groups based on chromosomal patterns. Those with a disomic chromosomal pattern or only p16 and/or chromosome 3 positivity were considered to be at low-risk (LR) for recurrence/progression, whereas patients with a chromosomal pattern including aberrations of chromosomes 7 and/or 17 were considered to be at high risk (HR). In total, 30/81 were classified as HR patients and 51/81 as LR patients according to the aforementioned FISH criteria. Patients were followed as per the previously detailed protocol (14) over the past 10 years. In brief, patients with a low-risk pattern underwent the FU of low-risk patients, which includes cytology and an ImmunoCyt/ucyt+ every 3 months and one cystoscopy every year, or cystoscopy in case of a doubtful/positive cytology or ImmunoCyt/ucyt+, while patients with a high-risk pattern were followed every 3 months with cytology, ImmunoCyt/ucyt+ and cystoscopy.

Statistical evaluation. Univariate analysis, using Mantel-Cox log-rank test for disease-free, progression-free survival and overall survival, was employed to determine the prognostic significance of FISH analysis. Disease-free survival time was calculated as the period between the date of first diagnosis and first recurrence according to the Kaplan-Meier product-limit method. Multivariate analysis using Cox proportional hazards regression model for both disease-free survival and overall survival was performed in order to determine prognostic significance of FISH and grade. Hazard ratios (HR) were expressed with 95% confidence interval (CI). A *p*-

Table I. *Histopathological and clinical data for 81 patients with intermediate risk urothelial cancer*

	Low-risk	High-risk
pTaG1 multifocal or >3 cm (n=22)	15	7
pTaG2 (n=53)	32	21
pT1G2 (n=6)	4	2
Female	8	3
Male	43	27

Mean age 69.9 years, range=41-94, median=71, SD 11.2

Table II. *Results of a Multicolour-FISH analysis in 81 under follow-up after patients with intermediate-risk urothelial carcinoma*

Low-Risk (n=51)	FISH-negative (disomic)	16
	p16/CEP3-positive	35
High-Risk (n=30)	CEP7/17-positive	30

Table III. *Recurrence/progression rate in 81 patients with low- and high-risk FISH pattern.*

Patients (n=81)	Recurrence	Progression	Total
Low-Risk (n=51)	14/51 (27.4%)	5/51 (9.8%)	19/51
High-Risk (n=30)	12/30 (40%)	6/30 (20%)	18/30

value<0.05 was set to be statistically significant. All data were analyzed with IBM SPSS Statistics, Version 20.

Results

Using the Multicolour-FISH analysis, 16 of 81 patients (19.8%) were negative (disomic pattern) and 65 of 81 patients (80.2%) were FISH-positive (aberration of any analysed probes). Out of FISH positive patients, 35 of 65 (53.8%) showed loss of one or both alleles of p16 and/or a positivity of chromosome 3. The low-risk group was formed by the 51 (62.9%) patients with either the disomic chromosomal pattern or only p16 and/or chromosome 3 positivity. The remaining 30 of 81 (37.1%) patients who had aneusomy of chromosome 7 and/or 17 formed the high-risk group (Table II). The mean observation time was 79.0 months (range=6-130 months, median=95, SD 40.7).

In total, 37 of 81 patients (45.7%) showed recurrent disease after a mean observation time of 48.9 months (range=3-130 months, median=105, SD 43.9). In patients with a low-risk chromosomal pattern, 19/51 (37.3%) recurred after an estimated mean observation time of 84.9 months (range=69.6-100.3, median=103, Standard Error (SE) 7.8). In detail, in the low-risk group, 16 FISH-negative patients (31.2%) displayed a histologically-verified recurrence, and 14/35 patients with p16 and/or chromosomes 3 positivity (40.0%) presented with recurrent disease. Five patients (9.8%) of this low-risk group showed progression. In the high-risk group the progression was 20% (6/30) (Table III).

In 18/30 (60.0%) patients with a high-risk pattern recurrence developed within an estimated mean observation time of 50.7 months (range=30.8-70.6, median=73, SE 10.2). The univariate Mantel-Cox log-rank test for disease-free survival showed significant differences between the low-risk group and the high-risk group for disease-free survival (chi-squared=7.807, p=0.005) (Figure 1) and for overall survival (chi-squared=4.297, p=0.038) (Figure 3); no significant result was obtained neither in progression-free survival (p=0.129), nor in cancer-specific survival (p=0.081) (Figure 2).

In addition, multivariate Cox proportional hazards regression model showed that FISH analysis significantly affected disease-free survival (p=0.006, HR 2.488) and overall survival (p=0.032, HR 2.040), independently from the grade of the tumor (p=0.228, HR 0.656 for DFS; p=0.100, HR=1.940 for OS) (Table IV).

Discussion

Tumor grade and stage are established prognostic factors for bladder cancer (1-3, 8-9). They are dependent on morphometric and morphological parameters. However, despite well-defined criteria for the diagnosis of UC, there is significant inter-observer variability especially in regards to tumor grade (15, 16). In addition, Ta and T1 stages include a heterogeneous group of tumours with variable recurrence and progression rates (9, 14, 17-19). Multiple studies show pTa urothelial carcinomas (UC) as having less of a propensity to progress (19) than pT1 tumours, which reach a progression rate between 30% and 50% (3, 4, 8, 9, 14, 18, 19). Unfortunately, little progress in sub-dividing these groups has been achieved in the last thirty years. There exists a need for additional parameters which allow for a more accurate assessment of the biological behaviour of the tumor.

The oncogenesis of pTa and pT1/Cis tumors appears to follow different pathways (4), possess different oncological potential and result in varying clinical outcomes. The loss of heterozygosity of chromosome 9p and 9q has been shown to be a crucial event in the transition of normal urothelium to papillary urothelial carcinoma. Previous studies from Krüger, Hitchings and Shariat (7, 19, 20) reported a worse prognosis and decreased disease-free survival in patients with a loss of p16 expression, which is located on the short arm of chromosome 9.

By using FISH in our previous studies (11, 14) we were able to assess the biological behaviour of intermediate urothelial carcinomas more accurately. This intermediate-risk group of urothelial carcinomas (10) was then sub-divided into a high and a low risk group based on the FISH analysis. Patients with a high-risk chromosomal pattern showed a significantly shorter disease-free survival time and higher progression rate compared to patients with a low-risk pattern. A significant difference (p=0.005) has been observed between the two groups. In addition, tumor recurrence in the low-risk group was nearly always of low grade and stage and represents less risk to the patient (20). Three out of 19 patients changed from pTa into pT1 but remained of low grade, 1/19 changed from low-grade to high-grade tumour. The follow-up scheme for intermediate risk patients was therefore modified at the Central Hospital of Bolzano according to their chromosomal pattern. Patients with a low-

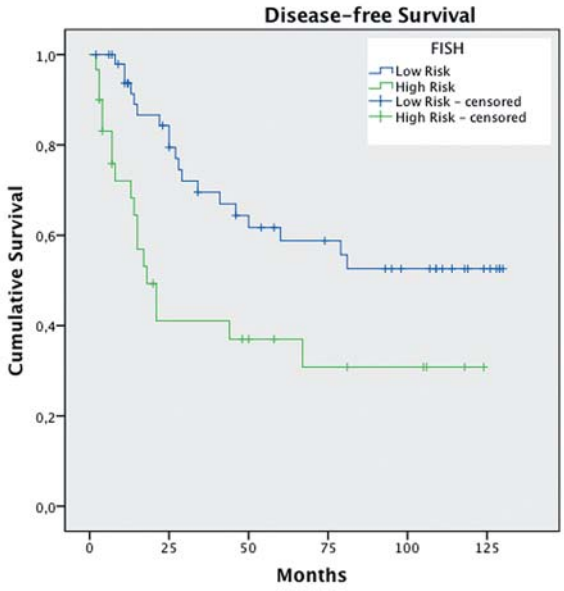

Figure 1. *Univariate Mantel-Cox log-rank test for disease-free survival for overall survival (Chi-square=7.807, p=0.005).*

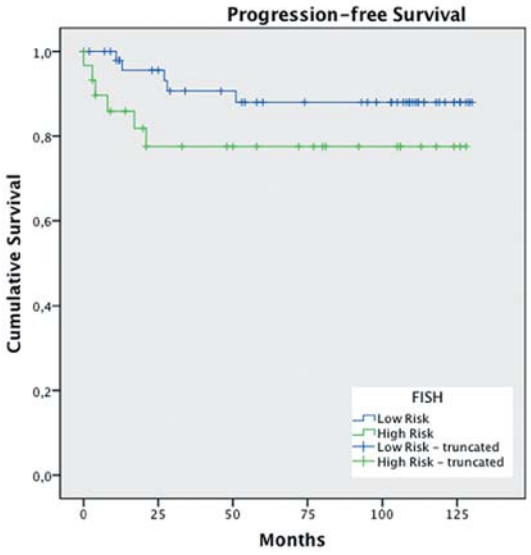

Figure 2. *Univariate Mantel-Cox log-rank test for progression-free survival for overall survival (Chi-square=2.301, p=0.129).*

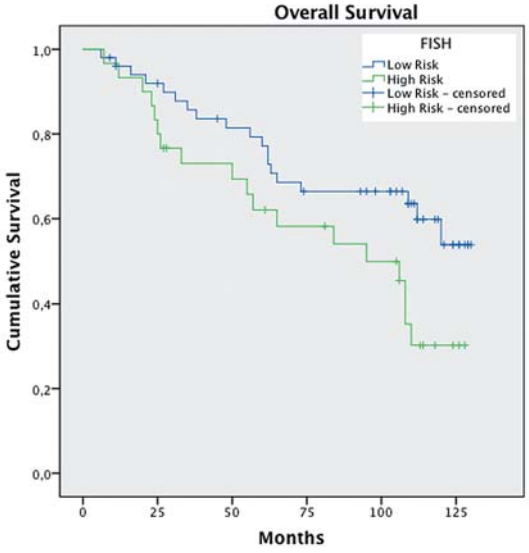

Figure 3. *Univariate Mantel-Cox log-rank test for overall survival (Chi-square=4.297, p=0.038).*

risk pattern underwent the FU of low-risk patients including cytology and ucyt+ every 3 months and one cystoscopy every year. Patients with a high-risk pattern were followed every 3 months with cytology, ucyt+ and cystoscopy.

In patients with a low-risk pattern, follow-up may be less rigorous and the number of cystoscopies can be safely reduced from 8 to 2 in the first two years. In fact, the recurrence rate was 37.2% in the low-risk group and occurred after a mean observation time of 84.9 months (range=69.6-100.3, SE 7.8). In contrast, in the high-risk group, there was a recurrence rate of 60%, which presented earlier at a mean of 50.7 months (range 30.8-70.6, SE 10.2). Furthermore the progression rate was twice as high in the high-risk group (20%) as it was in the low-risk group (10%).

However, overall and disease-free survival according to FISH analysis produced results statistically significant while cancer-specific survival was not statistically significant between the groups. This could be explained by the small number of patients who showed progression and died of disease but also by the fact that patients with urothelial cancer are generally of higher age and therefore have multiple morbidities which lead to death, not due to disease.

These results clearly show that the intermediate-risk group is a very heterogeneous group, which ranges from the biologically low-risk to high-risk tumors. This group does not reflect a cohort with a similar biological behaviour. Between two tumors in the same group the spread of risk is widely open and the clinical outcome can be totally different. The present study shows that a risk management of intermediate-risk UC based on the chromosomal pattern and on the biological behaviour of the tumor is reasonable, feasible, and safe. Its advantage is that it provides a more objective evaluation of tumor aggressiveness than that of other traditional stage and grading systems.

Using molecular and genetic knowledge of tumor biology according to FISH, we believe a risk stratification protocol can better-characterize the biological potential of the heterogeneous group of Ta, T1 and CIS bladder tumours. Our long-term follow-up data on intermediate-risk UC patients appears to be

Table IV. *Multivariate survival analyses*.

		B	SE	Wald	df	*p*-Value	OR	CI	
								Lower	Upper
Overall survival	FISH	0.713	0.332	4.607	1	0.032	2.040	1.064	3.912
	G	0.663	0.403	2.700	1	0.100	1.940	0.880	4.278
Cancer-specific survival	FISH	1.244	0.733	2.878	1	0.090	3.469	0.824	14.599
	G	1.292	1.074	1.447	1	0.229	3.639	0.443	29.862
Disease-free survival	FISH	0.912	0.332	7.532	1	0.006	2.488	1.298	4.772
	G	–0.421	0.349	1.451	1	0.228	0.656	0.331	1.302

G: Tumor grade; B: B-coefficient, SE: standard error, Wald: Wald coefficient, df: degree freedom; *p*-value: significance, OR: odds ratio, CI: Confidence interval.

able to divide tumors into low and high risk groups for recurrence, based on molecular/genetic changes observed with FISH. Further prospective studies are warranted on this matter.

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