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Differential Diagnosis and Evaluation of the Clinical Course of Transurethrally Resected T1G3 Urothelial Carcinoma of the Bladder by DNA Image Cytometry

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Abstract. *The value of DNA image cytometry in the differential diagnosis of 106 T1G3 urothelial carcinomas of the bladder and the long-term prognosis (recurrence-free interval, survival) of the patients was tested in comparison with Ta/T1G1 (n=30) and Ta/T1G2 carcinoma (n=54). Monolayer smears were prepared from three 50-µm-thick sections by a cell separation technique and were stained according to Feulgen. The DNA content of 250 epithelial cells, chosen at random, was determined using a TV-image analysis system CM-1 (Hund, Wetzlar, Germany). The DNA content of 30 lymphocytes served as an internal standard for the normal diploid value in every individual case. Different DNA cytometric parameters and the mean nuclear area were calculated. In comparison with G1- and G2-cases, the mean values of all DNA cytometric variables were markedly increased in the group of T1G3 cases, most obviously for the 5cEE, the mean ploidy and the ploidy imbalance (0.0006≥p≥0.0001). However, a remarkable overlay of the data distribution had to be considered. An aneuploid DNA stemline ploidy was highly characteristic for T1G3 urothelial carcinoma (sensitivity: 92%), but not sufficiently specific (57%). However, if increased values for the mean ploidy, the 2cDI, the 5cEE or the 9cEE (specificity: 86% - 89%) were present additionally, the diagnosis of a T1G3 urothelial carcinoma could be made cytometrically. Follow-up data for survival (recurrence) analysis was available for 90 (82) patients of the T1G3 group. Using the median value as threshold, significant differences in survival were found for the mean ploidy only (p=0.0353). The length of the recurrence-free interval was significantly different for the entropy (p=0.0205), the 2cDI (p=0.0309) and the mean ploidy (p=0.0442). In conclusion, DNA single cell cytometry represents a highly relevant tool in the objective identification of T1G3 urothelial carcinoma of the bladder, with a sufficient sensitivity and specificity. Further, this method enables prediction of tumor recurrence if suitable variables are chosen. The long-term survival of patients with T1G3 urothelial carcinoma can be estimated by DNA cytometry only in a limited manner, possibly due to the fact that the causes of death in the mostly elderly patients will be independent from the limited tumor disease.*

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The management of high-grade bladder tumors with invasion of the submucosal layer (T1G3-tumors) remains controversially, due to their marked biological propensity for recurrence in 70% to 80% of cases and for progression in 33% to 48% of cases, with consequences on patient survival (1-3). In recent years, organ-preserving approaches have been developed in addition to the concept of early cystectomy. Arguments for the latter method of treatment have been favored by reports of frequent clinical understaging in up to 40% of the cases. On the other hand, transurethral resection followed by adjuvant therapy, *i.e.* BCG instillation therapy, intravesical chemotherapy or combined adjuvant approaches, resulted in significantly decreased rates of local recurrence and progression (7.4%-30%) (3-7).

Table I. *Mean values and standard deviations of DNA image cytometric variables and the mean nuclear area.*

Variable	T1/Ta G1 (n=30)	T1/Ta G2 (n=54)	T1 G3 (n=106)
DNA stemline ploidy [c]	2.12±0.11	2.13±0.17	2.54±0.66
Mean ploidy [c]	2.30±0.27	2.74±0.44	3.75±0.75
2cDI	0.43±0.59	1.71±1.56	5.18±4.76
5cEE [n]	0.40±0.77	5.31±5.35	32.85±30.58
9cEE [n]	0.03±0.18	0.33±0.78	3.08±5.55
Entropy	2.82±0.55	3.66±0.67	4.46±0.49
Ploidy imbalance [%]	–53.03±32.68	–25.52±41.28	14.74±38.18
Mean nuclear area [μm^2]	41.95±9.69	5.25±7.82	49.00±10.22

Table II. *Pairwise comparison of the data distribution of DNA image cytometric variables and the mean nuclear area. P-values of the U-test.*

Variable	T1/Ta G1 *vs.* T1/TaG2	T1/Ta G1 *vs.* T1 G3	T1/Ta G2 *vs.* T1 G3
DNA stemline ploidy	> 0.05	0.0006	< 0.0001
Mean ploidy	< 0.0001	< 0.0001	< 0.0001
2cDI	< 0.0001	< 0.0001	< 0.0001
5cEE	< 0.0001	< 0.0001	< 0.0001
9cEE	0.0447	< 0.0001	< 0.0001
Entropy	< 0.0001	< 0.0001	< 0.0001
Ploidy imbalance	< 0.0001	< 0.0001	< 0.0001
Mean nuclear area	0.0418	0.0002	0.0163

Thus, to plan an effective therapy, objective data are highly recommended, based on tumor biological or morphometric methods. In various tissues, malignant transformation has been predicted by DNA image cytometry, including precancerous squamous epithelial lesions of the oral (8), laryngeal (9), bronchial (10), vulvar (11) or cervical (12, 13) mucosa or the skin (14), borderline lesions of the ovary (15), gastric dysplasia (16), hyperplastic endometrial lesions (17) or precancerous lesions of the endocervical glands (18). In urothelial carcinoma, recent DNA cytometric reports focus mainly on G1 cases in order to come to an early diagnosis of primary or recurrent tumor (19-21). However, data on the relevance of the method concerning the special problem of T1G3 tumors are still lacking.

Our study, on 106 cases of locally resected T1G3 urothelial carcinoma of the bladder, revealed a high sensitivity for the detection of DNA aneuploidy in T1G3 urothelial carcinoma as compared to the control group of G1- or G2-tumors, respectively. The prognostic significance concerning the rate of recurrence was more obvious than concerning the length of survival.

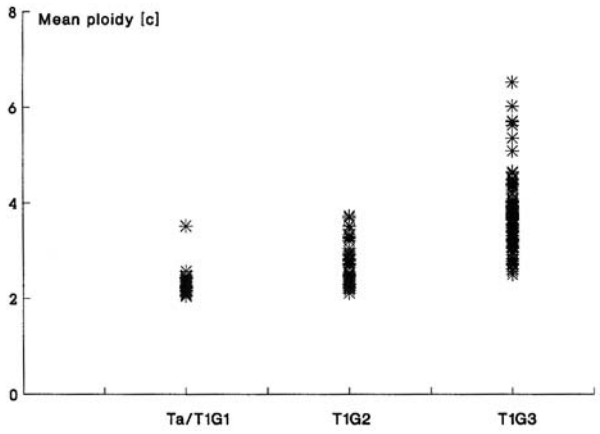

Figure 1. *Data distribution of the mean ploidy values for Ta/T1G1- (n=30), Ta/T1G2- (n=54) and T1G3-cases (n=106) of urothelial carcinoma of the bladder.*

Materials and Methods

Patients. One hundred and six specimens of poorly-differentiated urothelial carcinoma, staged as invasive in the submucosal layer only (T1G3) on transurethral resections (TUR), were investigated. As T1G3-cases are rare, the cases were collected from the files of all of the four Departments of Urology involved in this study. Thirty TUR-specimens of well-differentiated urothelial carcinoma, staged as papillary non-invasive or as invasive in the submucosal layer (TaG1 / T1G1) and 54 TUR-specimens of moderately-differentiated urothelial carcinoma of the same staging groups (TaG2 / T1G2), collected consecutively from the files of the Institute of Pathology, University of Aachen, Germany, served as disease control groups.

The mean age of all the 190 patients was 68.3±10.4 (SD) years; 150 were male and 40 were female. There was no statistically significant difference in the distribution of age and sex between the three patient groups ($p>0.05$).

Methods. On the paraffin blocks, the region of interest was marked in order to prevent measurements on tissue areas beside the lesion. Then, monolayer smears were prepared from three 50-μm-thick sections of this region by a cell separation technique (22, 23) and were stained according to Feulgen, as described in detail previously (24). Briefly, acid hydrolysis (4N HCl, 27.5˚C, 55 minutes) was followed by a 60-minute incubation in Schiff's reagent at room temperature.

For DNA quantification, per nucleus the integrated optical density and the nuclear area were measured at a wavelength of 570 nm (halfwidth of the interference filter:±10 nm) using a TV-image analysis system CM-2 (Hund, Wetzlar, Germany) (25). The mean integrated optical density of 25-30 lymphocytes served as an internal standard for the normal diploid value in every individual case (error of the mean of the reference cell population: <3%). The results were documented in a DNA frequency histogram.

From the distribution of the single values, the mean nuclear area and various DNA cytometric variables were calculated

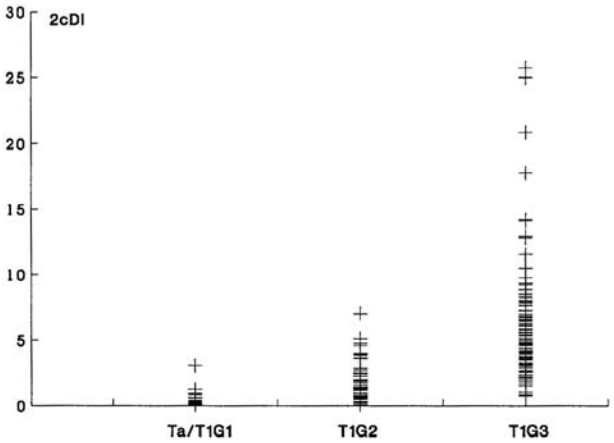

Figure 2. *Data distribution of the 2cDI values for Ta/T1G1- (n=30), Ta/T1G2- (n=54) and T1G3-cases (n=106) of urothelial carcinoma of the bladder.*

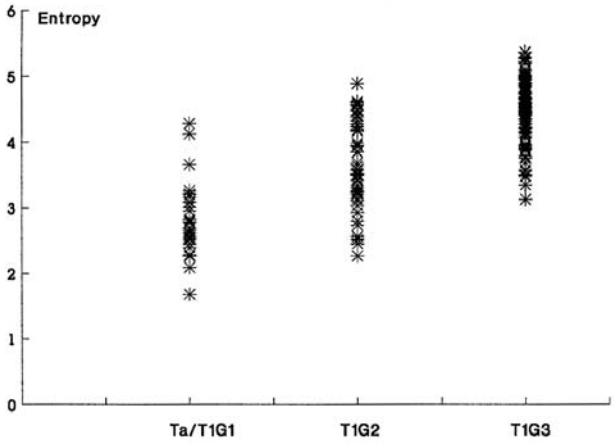

Figure 3. *Data distribution of the entropy values for Ta/T1G1- (n=30), Ta/T1G2- (n=54) and T1G3-cases (n=106) of urothelial carcinoma of the bladder.*

following the proposals of the recent ESACP consensus report (26), including the DNA stemline ploidy (27), the mean ploidy, the 2c deviation index (28), the 5c- and the 9c exceeding events (5cEE, 9cEE) (29, 30), the entropy (31) and the ploidy imbalance (17).

Statistics. For the comparison of the data sets of the three series of tumors, the Mann-Whitney *U*-test was used in order to analyze the data distribution. Multi-field tables were interpreted using the Pearson Chi^2-test of independence. Univariate survival analysis or the analysis of the recurrence-free interval, respectively, were performed according to Kaplan and Meier (32). The respective curves were tested on significant differences by a pairwise Wilcoxon-Breslow test. Statistical significance was accepted for $p<0.05$.

Table III. *Sensitivity and specificity of DNA image cytometric variables and the mean nuclear area for the differential diagnosis of T1 urothelial carcinoma of the bladder. "Positive" group=106 T1G3-cases. "Negative" group=84 T1/Ta-cases, graded as G1 or G2.*

Variable	Threshold	Sensitivity	Specificity
DNA stemline ploidy [c]	2.20	92% (97/106)	57% (48/84)
Mean ploidy [c]	3.20	78% (83/106)	87% (73/84)
2cDI	3.06	79% (84/106)	86% (72/84)
5cEE	10	79% (84/106)	89% (75/84)
9cEE	0	62% (66/106)	87% (73/84)
Entropy	4.20	75% (79/106)	81% (68/84)
Ploidy imbalance [%]	-11	73% (77/106)	79% (66/84)
Mean nuclear area [µm2]	47.0	59% (63/106)	62% (52/84)

Table IV. *Survival analysis and analysis of the recurrence-free interval. With the exception of the DNA stemline ploidy with a defined threshold at 2.20c (non-aneuploid vs. aneuploid DNA stemline ploidy), the median values of the data distribution of the 90 (82) cases with follow-up were used as thresholds between "low values" and "high values". Statistical analysis was performed using the Wilcoxon-Breslow-test.*

Survival analysis

Variable	low values		high values		*p*-value
	5-year probability	mean [months]	5-year probability	mean [months]	
DNA stemline ploidy	57.1%	74	61.2%	88	> 0.05
Mean ploidy	72.0%	96	47.7%	71	0.0353
2cDI	65.4%	89	54.1%	79	> 0.05
5cEE	60.6%	89	55.1%	77	> 0.05
9cEE	63.1%	85	55.2%	83	> 0.05
Entropy	67.1%	96	52.3%	66	> 0.05
Ploidy imbalance	60.2%	79	59.1%	84	> 0.05
Mean nuclear area	57.4%	84	62.0%	81	> 0.05

Analysis of the recurrence-free interval

Variable	low values		high values		*p*-value
	5-year probability	mean [months]	5-year probability	mean [months]	
DNA stemline ploidy	71.0%	77	74.1%	104	> 0.05
Mean ploidy	86.0%	100	55.4%	65	0.0442
2cDI	89.0%	102	54.8%	66	0.0309
5cEE	85.3%	104	59.4%	69	> 0.05
9cEE	84.5%	102	55.7%	66	> 0.05
Entropy	87.2%	116	59.4%	66	0.0205
Ploidy imbalance	83.8%	96	61.9%	74	> 0.05
Mean nuclear area	80.8%	92	63.3%	80	> 0.05

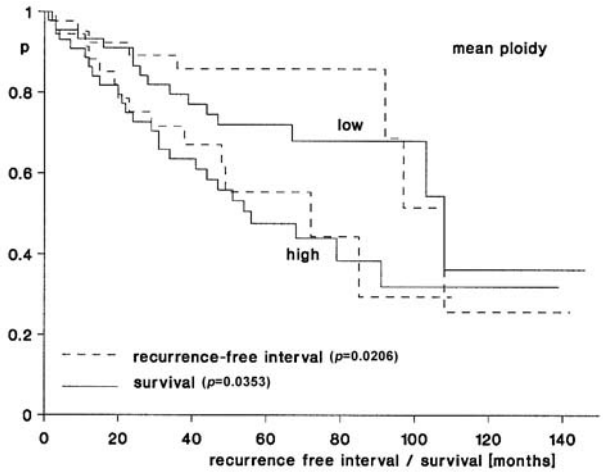

Figure 4. *Kaplan-Meier curves for survival analysis and analysis of the recurrence-free interval of T1G3 urothelial carcinoma for the mean ploidy.*

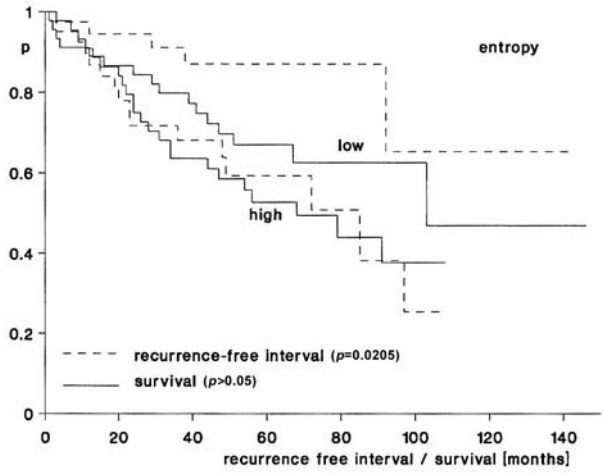

Figure 6. *Kaplan-Meier curves for survival analysis and analysis of the recurrence-free interval of T1G3 urothelial carcinoma for the entropy.*

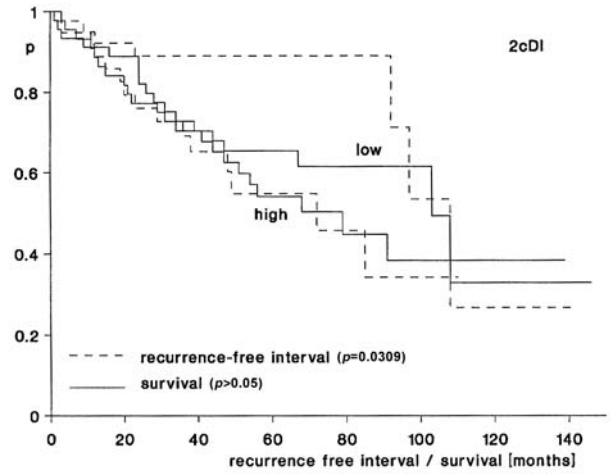

Figure 5. *Kaplan-Meier curves for survival analysis and analysis of the recurrence-free interval of T1G3 urothelial carcinoma for the 2cDI*

Results

Data distribution. The mean values and the standard deviations of the various DNA cytometric variables and of the mean nuclear area are presented in Table I. In comparison with G1- and G2-cases, the mean values of all DNA cytometric variables were markedly increased in the group of T1G3-cases, most obviously for the 5cEE, the mean ploidy and the ploidy imbalance. The mean value of the mean nuclear area was approximately identical for all three groups. The differences for the DNA cytometric variables were statistically significant in the *U*-test at a *p*-level <0.0001 (Table II), with the only exception being the DNA stemline ploidy comparing G1- and G3-cases ($p=0.0006$). Also, the mean nuclear area values were different between G1- and G3-cases ($p=0.0002$) and G2- and G3-cases ($p=0.0163$). For all variables, however, a remarkable overlay of the data distribution has to be considered. Comprehensive examples are presented in Figures 1 to 3. After stratifying the data into two groups according to the median value, multi-field tables revealed statistically significant differences in the Pearson Chi²-test as well for the DNA cytometric variables ($p<0.0001$) and for the mean nuclear area ($p=0.0018$).

Diagnostic data interpretation. For diagnostic data interpretation, the cases of G3-carcinoma were defined as the test group, the cases of G1- and G2-carcinoma as the control group. From the data presented in Table III, it may be concluded that an aneuploid DNA stemline ploidy is highly characteristic of T1G3 urothelial carcinoma (sensitivity: 92%), but not sufficiently specific (57%). However, if additionally increased values for the mean ploidy, the 2cDI, the 5cEE or the 9cEE (specificity: 86% - 89%) are present, the diagnosis of a T1G3 urothelial carcinoma should be favored.

Survival analysis and analysis of the recurrence-free interval in the T1G3 cases. In the group of the T1G3-cases, follow-up data for survival analysis was available for 90 patients; additionally, data about the recurrence-free interval were provided for 82 patients: 68 of them had been treated in Verona (76%), and 11 patients each in Munich (12%) and in Aachen (12%). For 8 of the 11 patients from Munich, no data about the recurrence-free interval could be obtained.

The observation periods were not significantly different between the three institutions (*U*-test: $p>0.05$). At the end of the observation period, 49 patients were alive after 70.0±26.6 (SD) months. Forty patients had died after 34.6±27.2 (SD) months. One patient had been lost from follow-up after 2 months. Sixty patients were recurrence-free for 42.7±31.0 (SD) months, while in 22 patients tumor recurrence had been diagnosed after 36.6±33.3 (SD) months. In Kaplan-Meier curves, significant differences in survival between two groups of patients (1:1 distribution; threshold: median value of the data distribution of the T1G3 group, Table IV) were found for the mean ploidy only ($p=0.0353$, Figure 4). 2cDI and entropy revealed survival differences in the survival curves, which were not statistically significant ($p>0.05$, Figures 5, 6). No differences in survival became obvious for the remaining DNA cytometric variables and for the mean nuclear area, either in the survival curves, or statistically ($p>0.05$). The length of the recurrence-free interval (Table IV) was significantly different for the two groups of patients for the entropy ($p=0.0205$), the 2cDI ($p=0.0309$) and the mean ploidy ($p=0.0442$) (Figures 4-6). 5cEE and 9cEE revealed differences in the recurrence-free interval in the respective curves, which were not statistically significant ($p>0.05$). No differences in the recurrence-free intervals were found for the remaining DNA cytometric variables and for the mean nuclear area. In conclusion, especially DNA cytometric variables reflecting the DNA data distribution as a whole (entropy, mean ploidy, 2cDI) were suitable to predict the patient's risk of local tumor recurrence or the patient's survival prognosis. Those variables which focus on single event detection (5cEE, 9cEE, ploidy imbalance) or on the position of the modal value of the DNA data distribution (DNA stemline ploidy) were of only minor meaning. The mean nuclear area failed to give prognostic information.

Discussion

Therapy in T1G3 urothelial carcinoma of the bladder remains controversial to date (1-7), ranging from bladder-conserving local surgical therapy, often followed by BCG instillation, to early cystectomy. This wide range of therapeutic options requires additional tumor biological data, as the therapeutic decision has to be taken as objectively as possible. In our study, DNA image cytometry was applied for the first time, to the best of our knowledge, systematically on T1G3 urothelial carcinoma of the bladder. Our results, obtained in comparison with G1- and G2-tumors, underline the diagnostic power of DNA image cytometry in urothelial carcinoma. It could be demonstrated that various DNA cytometric variables reveal significant differences between G3-tumors and G1-/G2-tumors, which indicate, that DNA cytometry may be useful in the objective classification of tumors as poorly-differentiated, with an approximately 80% sensitivity and a 80% specificity.

However, the prognostic meaning of this method should be discussed. After stratification of the patients into two groups of "low" and "high" levels using the median value as threshold, it could be demonstrated that particularly tumor recurrence can be predicted by DNA cytometry, if suitable variables are chosen. Those variables that reflect the DNA distribution pattern as a whole (2cDI, entropy, mean ploidy) revealed statistical significance. The stemline ploidy, to be interpreted as an indicator of the modal value of the DNA distribution, or variables that focus on single event detection (5cEE, 9cEE, ploidy imbalance), were of only minor or even no significance, although some of them showed a useful prognostic trend.

The long-term survival of patients can be estimated by DNA cytometry only in a limited manner. In principle, the mean ploidy, the entropy or the 2cDI revealed better results than the other variables; however, only for the mean ploidy could a statistically significant result be obtained. Most probably this finding reflects the fact that, in many cases, the death of the mostly elderly patients was independent of the urothelial carcinoma of the bladder. Although the concrete cause of death could not be analyzed, it may be concluded from the average age of approximately 68 years that several patients will have suffered from cardiac diseases, tumors of different sites of the body or further severe diseases. Thus, the occurrence of a limited urothelial carcinoma (T1) might have been of only minor prognostic meaning.

In conclusion, our investigation, applying various DNA cytometric variables, gives further evidence that DNA image cytometry is of high value in the prediction of recurrence in cases of conservatively-treated T1G3 urothelial carcinoma of the bladder. Thus, the results of our study justify the hypothesis that DNA single cell cytometry in high-grade urothelial carcinoma could be as relevant as it has been demonstrated for low-grade urothelial carcinoma and for precancerous or cancerous conditions of squamous epithelium (8-14) or glandular epithelium (15-17) of various sites.

Future studies could test whether the combination of DNA image cytometry with immunohistochemistry or molecular methods could lead to an improvement of the diagnostic or prognostic stratification of the patients. One candidate could be the proliferation marker MIB-1. In a study of 35 patients, whether MIB-1 immunostaining as a surrogate for the mitotic rate could predict response to BCG intravesical therapy in patients with completely resected T1G3 urothelial carcinoma of the bladder was tested (33). That patients with a MIB-1 rate <20% showed no tumor recurrence indicates a possible future use of MIB-1 in this tumor entity; on the other hand, MIB-1 immunostaining could, unfortunately, not predict BCG

therapy failure and, thus, was of only limited clinical significance. Another marker, that has been quite popular in recent years, is p53. However, in a study on 29 cases of T1G3 urothelial carcinoma, it became obvious that an immunohistochemically-detected overexpression of p53 is of no predictive value for recurrence and progression after treatment with intravesical BCG (34). In the field of molecular pathology, the use of the multitarget FISH probe UroVysion and of image cytometry as supplementary methods, that have been tested recently for the detection of G0- and G1 carcinoma (21), could possibly be used in a modified way also to analyze the potential of high-grade urothelial carcinomas for the prediction of recurrence or prognosis. The application of this method, however, requires the identification of molecular targets that are concretely associated with the process of tumor progression.

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