QUANTICYT: KARYOMETRIC ANALYSIS OF BLADDER WASHING FOR PATIENTS WITH SUPERFICIAL BLADDER CANCER

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ABSTRACT

Objectives. Quantitative cytology by image-analysis techniques enables objective interpretation of nuclear features in light microscopic images. QUANTICYT, a quantitative karyometric cytology system, was used in the follow-up of patients with superficial bladder cancer.

Methods. From 1992 to 1995, 4137 samples from 1412 patients were obtained. At 1-year follow-up after the initial bladder washing, a tumor recurrence rate of 21% was found. In this period, tumor progression to invasive disease occurred in 1.6% of patients. Scoring of tumor by the QUANTICYT system was based on two nuclear features: the 2c deviation index and the mean of a nuclear shape feature: MPASS.

Results. The method was found to be reproducible and superior to visual cytologic interpretation. QUANTICYT analysis of the bladder washings resulted in a score of low, intermediate, and high risk. In a multivariate analysis, highest grade of earlier tumor and QUANTICYT risk score were the best predictors of tumor recurrence and progression. For the easy application of QUANTICYT analysis in daily routine, a report form that included patient history and DNA histogram was developed.

Conclusions. QUANTICYT karyometric analysis of bladder-wash material proved a useful, clinically applicable grading tool in the follow-up of patients with superficial bladder cancer, with sufficient power to be used in decision-making in the individual patient.

In approximately 66% to 80% of patients with bladder cancer, the disease starts as a superficial (that is, noninvasive) lesion in the bladder mucosa.1,2 These so-called Ta and T1 tumors show a tendency to recur in 30% to 60% of patients during follow-up after treatment of the primary tumor.2,4 Moreover, 15% of patients finally suffer invasive, often lethal, forms of bladder cancer.3

In recent years, clinical markers for the prediction of prognosis have been studied.3,3 Multiple and early recurrent cancers showed a high tendency to progress to invasive disease.3 Kurth et al.3 presented a table for relative progression-risk estimation using tumor grade, tumor size, and early recurrence rate. Relative risk values for progression ranged from 0 in small (less than 1.5 cm) G1 tumors with a recurrence rate per year of less than 1, to 3.7 in large (greater than 3.0 cm) G3 cancers that were frequently recurrent (more than three times per year).3 Patients were divided into three risk groups; 3-year progression rates per group were 7%, 16%, and 31% respectively.3

The follow-up of patients with superficial bladder cancer is done by cystoscopy and cytology. Hall et al.6 proposed a follow-up scheme based on the initial clinical prognostic findings as found by Kurth et al.3 Cystoscopy frequency in patients at low risk for tumor progression could be significantly reduced.6 So far, however, these recommendations have not been applied on a wide scale.

However, even in the low-risk group of patients (as defined by Kurth et al.3), 7% of tumors still progress to invasive disease within 3 years after
diagnosis; this group comprised 52% of their patient population, whereas the high-risk group 3 comprised only 6.7% of the patients (of whom 42% progressed). Hence, the absolute number of progressive tumors in the low-risk group 1 is still higher than the absolute number in the high-risk group 3. These data indicate that changing treatment and follow-up schemes based on relative risk alone underestimates the potential risk of the less-aggressive but more frequent cancers; this may put an equal number of patients at risk of late detection of progression as are found in time by meticulous follow-up of the high-risk patient group. Two factors are important to overcome this dilemma: (1) more accurate prognostic markers, and (2) a reliable tool for the monitoring of bladder mucosal changes during patient follow-up, particularly in the less-progressive cancers.

Cytology methods have been used in the detection and follow-up of bladder tumors. Visual microscopic interpretation of material, however, has been subject to low inter- and intraobserver reproducibility. Light-microscopic image analysis of cytologic material allows ploidy analysis as well as objective interpretation of cellular and nuclear features reflecting malignant deformation. In earlier studies, the use of a quantitative cytology system (QUANTICYT) for bladder-wash material was described. In the present study, the QUANTICYT system is tested for patients with superficial bladder carcinoma: (1) for diagnostic value (whether the QUANTICYT score correlates with the presence of tumor in the bladder and (2) for its ability to predict prognosis in superficial bladder cancer.

MATERIAL AND METHODS

PATIENTS

From October 1990 to March 1995, 4137 bladder-wash samples from 1412 patients in six urologic institutes (one academic and five nonacademic) were obtained. Patients diagnosed with a superficial (stage smaller than pT2) transitional cell carcinoma of the bladder were eligible for the study. For each bladder-wash sample, the cytostoscope findings (multiplicity, tumor size, and papillary or solid type lesions) were documented. Moreover, patient history concerning earlier tumor recurrences and related treatment modalities were considered. When histologic samples were taken, these data were also entered.

The bladder-wash material was obtained by rinsing the bladder at least twice with saline solution, either through a catheter or a cystoscope. Then, 25 cc of the material was instantly fixed in 25 cc of 50% ethanol containing 2% polyethylene glycol (Carbowax) and stored at 5°C. Bladder washings were always performed after emptying of the bladder and prior to intravesical manipulations, instillations, and tumor resection. At the laboratory, the material was centrifuged (2000X for 10 minutes), and the pellet resuspended in Carbowax fixative. For staining, the material was centrifuged with a Cytospin device (Cytospin, Shandon) on gelatin-coated slides, postfixed with Böhm fixative (85% methanol, 10% buffered formalin, 5% glacial acetyl acetic acid), and Feulgen stained (5 N HCl for 60 minutes; Schiff-reagent, Merck, Germany for 30 minutes, both at room temperature).

THE QUANTICYT SYSTEM

The karyometric analysis was performed on the Feulgen-stained slides using a personal-computer-based image analysis system (Fig. 1). On each slide, 50 randomly selected images containing 100 to 500 nuclei were analyzed after recording on the hard disk of the computer. Recording of these images took 2 minutes per slide. The 50 images per slide were automatically analyzed, without intervention by the technician, in 50 to 80 minutes. After the automatic analysis, the selected nuclei were contoured and numbered in the images, thus enabling post-analysis visual verification of the objects. Lymphocytes in the material were used as reference for 2c deoxyribonucleic (DNA) content. For each nucleus present in the images, the DNA content and nuclear shape feature PASS (based on the smoothed Freeman difference chain code) were determined. In each sample, the 2c deviation index (2cDI) according to Böcking et al.11 was calculated. Both features were used to determine the QUANTICYT diagnosis as described earlier. The QUANTICYT scoring spectrum consisted of low risk, intermediate risk, and high risk, as was earlier determined by correlation with histology data.

Because several technicians recorded and reviewed the images, reproducibility of the karyometric analysis was tested in a set of 40 samples among three technicians. To compare cytologic and karyometric grading, 104 samples were interpreted by both techniques.

In 200 of 4137 samples (5%), QUANTICYT karyometric analysis could not be performed because of too few urothelial cells (at least 100 nuclei should be measured) or because of cystitis, in which case the large number of leukocytes may obscure the urothelial cells.

A database was linked to the system for documentation of patient-related information, such as earlier treatments and histology data. This information was provided by the urologist and filled out on the application form. Finally, a report form with clinical patient information and data concerning all samples of the patient present in the system was drawn (Fig. 2).

Statistical analysis was performed using the SPSS/PC+ software. Follow-up results are presented applying the Kaplan-Meier method; for significance, the log-rank test was used. Multivariate regression analysis according to Cox was performed. Significance of a test was assumed when P < 0.05.

RESULTS

REPRODUCIBILITY

The correlation of analysis of nuclear profile area, number of analyzed nuclei per sample, nuclear shape (PASS), and the 2cDI was tested among three technicians. The correlation values (Pearson r) between the measurements obtained by the different technicians range from 0.801 to 0.925 (P < 0.01). All correlations were significant and larger than 0.80, except for the 2cDI. This was caused by the large number of samples with 2cDI values smaller than 1. In this group, low correlation values among the measurements obtained by
the three technicians were found. In the range of 2cDI values larger than 1, \( r = 0.78 \). This is of minor importance in routine application because distinction between low 2cDI values (less than 1.0) is of no clinical importance. The random selection of images could cause differences in the analyzed number of nuclei per sample between technicians. Correlation of the number of nuclei analyzed, however, was high for all three technicians (\( r = 0.82 \)).

**Cytology and Karyometric Analysis**

To compare the sensitivity and specificity of the karyometric analysis with visual cytology, a subset of 104 random chosen samples was analyzed with both techniques. Due to inadequate material (too few diagnostic cells), 8 samples were not suited for cytologic analysis. In Table I, cytologic and karyometric analyses are compared. Cytology was graded as normal, low-grade, or high-grade malignancy.

To compare it with QUANTICYT analysis for the detection of tumor, low- and high-grade malignant cytology was considered positive for tumor, whereas tumor was assumed in the QUANTICYT analysis when this was intermediate- or high-risk. Sensitivity for cytology and for karyometric analysis was 71.6% and 95.2%, respectively, in this population; specificity was 60% and 65% for both techniques, respectively. Of the false-negative samples in cytology, 43% (10 of 23) were tumors with a histologic grade higher than Grade 1. The false-negatives in the karyometric analysis consisted of 3 grade 1 and 1 grade 2 tumor.

**Instillation Treatment and QUANTICYT Score**

The influence of intravesical therapeutic instillations on (bladder wash) cytology has been described.\(^{12,13}\) Bacillus Calmette-Guérin (BCG) instillations resulted in an increased number of polyploid cells,\(^{14,15}\) whereas mitomycin-C (MMC) treatment gave rise to cytologic abnormalities several months after instillation.\(^{12,13,16}\) The bladderwash samples taken during or 2 months after intravesical therapy were separately analyzed. The percentage of recurrent tumors (19%) during follow-up in this group (\( n = 155 \)) was similar to the entire population. All patients in the intravesical instillation group who developed a recurrent tumor had abnormal QUANTICYT evaluations (that is, intermediate or high risk).

**Prediction of Tumor Recurrence and Progression**

During a median follow-up of 18 months (range 2 to 56), 282 of 1412 patients (20%) developed a tumor recurrence. Progression to invasive disease was found in 30 of 1412 patients (2%). The QUANTICYT risk groups (low, intermediate, and high) correlated with tumor recurrence (Fig. 3; Table II). Although only 2% of patients had tumor progression, samples with
low- and intermediate-risk scores were significantly less likely to be followed by tumor progression than were high-risk-scored samples (Fig. 4; Table II). Of the clinical features, the presence of earlier bladder tumors was significantly correlated with a higher recurrence rate ($P < 0.01$, log rank) as compared with patients without earlier bladder cancer. In case of a lesion detected by cystoscopy a significantly higher recurrence rate ($P < 0.01$, log rank) was found for multiple lesions compared with solitary lesions (Fig. 5). There was no difference in recurrence rate between solid and papillary lesions detected by cystoscopy ($P > 0.10$, log rank), whereas tumor grade was only initially correlated with a higher recurrence rate (Fig. 6). Data on the univariate analysis of time to recurrence and progression for clinical and QUANTICYT scores are presented in Tables III through VI.

Multivariate Cox regression analysis for the prediction of tumor recurrence and progression showed the combination of highest grade of earlier tumors and the QUANTICYT risk group to be the
TABLE II. Tumor recurrence and progression rates after a
bladder-wash sample with a low-, intermediate-, or high-risk
QUANTICYT score (Q-score) (n = 1412)

<table>
<thead>
<tr>
<th>QUANTICYT</th>
<th>Recurrence (Progression) Rate (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low risk (n = 745)</td>
<td>0.54 (0.26) 2.82 (0.26) 5.50 (0.54)</td>
</tr>
<tr>
<td>Intermediate risk (n = 399)</td>
<td>2.75 (0.50) 8.02 (0.75) 12.53 (1.25)</td>
</tr>
<tr>
<td>High risk (n = 268)</td>
<td>5.97 (2.61) 13.06 (4.48) 20.52 (6.34)</td>
</tr>
</tbody>
</table>

* P < 0.001, log rank test, for both recurrence and progression.

FIGURE 4. Progression per risk group (n = 1412).

FIGURE 5. Recurrence rates in patients with solitary and multiple lesions detected by cystoscopy.

best predictors of recurrence, as well as tumor progression (Tables IV and VI).

COMMENT

Bladder-wash material proved to be superior to urine for routine cytology as well as for quantitative analysis. Flow cytometric studies showed that ploidy analysis of bladder-wash material is a sensitive method of tumor detection. Flow cytometry does not enable simultaneous visual interpretation and selection of cells. Moreover, flow cytometry is not suited for morphometric analysis. Quantitative light microscopic techniques can be used for DNA content analysis, as well as for morphometric analy-
s. Koss' group in Montefiore Medical Center in New York has extensive experience with an image analysis system for the classification of cells in voided urine. Sensitivity of this system was particularly high for high-grade tumors, but low-grade lesions were less readily diagnosed. The cell-to-cell reference classification and the use of voided urine instead of bladder-wash material are two possible reasons for the low diagnostic yield.

In bladder-washing material, two nuclear features were found in a multivariate analysis to correlate with histologic tumor grade: the 2cDI and the mean of a nuclear shape feature (MPASS). The 2cDI is a feature related to the difference of nuclear DNA content within a cell population. When nuclear DNA content shows a high variation between nuclei in one sample (for example, in aneuploid or polyploid samples or in samples with a high percentage of S-phase or G2M-phase nuclei), the 2cDI value is high. Of additional predictive value to the 2cDI was the MPASS feature value per sample. The PASS is a nuclear shape descriptor based on analysis of the smoothed Freeman difference chain code. In particular, PASS is sensitive to elongation of the nuclear shape. Low MPASS values indicate more ellipsoid-shaped nuclei. These two nuclear features, the 2cDI and the MPASS, are plotted in a scattergram that allows the comparison of subsequent samples of the sample patient in one plot.

Hence, the graphic representation of several samples makes this method particularly suitable for close monitoring of changes in the bladder-wash cytology profile of the patient in daily practice. There have been few reports of clinically applicable karyometry systems. However, findings on small patient populations using the karyometric system showed that it could provide potential clinical information. Thus, a clinically applicable system for the quantitative analysis of bladder-wash material was designed (QUANTICYT).

The findings of the present study show that QUANTICYT karyometric analysis aids in predicting tumor recurrences and progression in patients with superficial bladder cancer, with either abnormal or normal cystoscope findings. When no bladder abnormalities were found with the cystoscope, the presence of earlier bladder cancer was an important prognostic clinical feature. The prognostic value of prior recurrences was also found by Kurth et al. One-year recurrence rates were 3% in patients with low-risk QUANTICYT score and a primary tumor, whereas they were 34% in patients with high-risk QUANTICYT score and a history of recurrent bladder cancer. Remarkably, the presence of prior tumor recurrences was only of additional prognostic value in low- and intermediate-risk samples.

Not all bladder-wash samples were obtained when the tumor was present in the bladder. Hence, a subgroup of patients in whom a tumor was resected just after bladder-wash material sampling was studied (n = 232). Multivariate Cox regression analysis showed that tumor grade and QUANTICYT risk group were the best predictors of tumor recurrence in this patient group. The fact that QUANTICYT analysis is of prognostic value when done prior to transurethral tumor resection, as well as during follow-up, indicates that it can be a tool for longitudinal follow-up. Changes in QUANTICYT score during follow-up seem to indicate alterations in the bladder mucosa that subsequently influence prognosis.

In the present analysis, tumor progression occurred at low overall frequency (2.6%). No significant difference for progression rates was observed between low and intermediate QUANTICYT risk samples. The QUANTICYT score of high risk was strongly correlated with tumor progression. Whether the higher recurrence rate observed in intermediate-risk samples, as compared with low-risk samples, results in higher progression rates remains to be established by longer follow-up.

The false-positive rate of karyometric analysis during and shortly after intravesical chemotherapy or BCG instillations was increased compared with samples not taken during instillations. However, longer follow-up demonstrated that recurrences could still be predicted. In particular, aneuploidy was related to recurrent tumor. These data indicate that QUANTICYT analysis may still provide prognostic information during intravesical therapy, whereas routine cytology is considered unreliable in these patients.

TABLE V. Univariate analysis for the prediction of tumor progression (n = 1412)

<table>
<thead>
<tr>
<th>Prognostic Factor</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Age</td>
<td>0.9513</td>
</tr>
<tr>
<td>Sex</td>
<td>0.5962</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>0.4098</td>
</tr>
<tr>
<td>Tumor growth (solid/papillary)</td>
<td>0.1100</td>
</tr>
<tr>
<td>Highest stage of earlier tumor</td>
<td>0.9513</td>
</tr>
<tr>
<td>Highest grade of earlier tumor</td>
<td>0.0437</td>
</tr>
<tr>
<td>QUANTICYT risk group</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

TABLE VI. Multivariate analysis for the prediction of tumor progression (n = 1412)

<table>
<thead>
<tr>
<th>Prognostic Factor</th>
<th>(\beta)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>QUANTICYT risk group</td>
<td>1.4925</td>
<td>0.0001</td>
</tr>
<tr>
<td>Highest grade of earlier tumor</td>
<td>0.3055</td>
<td>0.0404</td>
</tr>
</tbody>
</table>
Cystoscopy is an important tool for follow-up in patients with superficial bladder cancer. Recently, the selection of which patients should undergo this invasive investigation was under analysis. The identification of patients at higher risk for tumor recurrence or progression was based on multiplicity and the presence of tumor recurrence at 3 months after transurethral resection. QUANTICYT in combination with these clinical data, aids in defining patients at risk, and in need for frequent cystoscopy. Prospective analysis of this risk factor-based follow-up is needed to incorporate it in daily routine. Whether QUANTICYT analysis can indicate patients likely to benefit from intravesical therapy remains to be established.

CONCLUSIONS

Quantitative light-microscopy techniques enable objective interpretation of nuclear shape and DNA content. The QUANTICYT score thus calculated from bladder-wash material correlates with tumor recurrences and progression in patients with superficial bladder cancer. Overall recurrence rates at 1 year for the QUANTICYT scores were 5.5% for low-risk samples, 12.5% for intermediate risk, and 20.5% for high risk. Progression rates were 0.5%, 1.3%, and 6.3%, respectively. In a multivariate analysis, karyometric data provide significant additional information to tumor grade for the prediction of tumor recurrence and progression. The QUANTICYT system enables monitoring of bladder mucosal changes in a quantitative and easy-to-read format. Selecting patients based on risk analysis may reduce the number of cystoscopies.

REFERENCES

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