Improved objectivity of grading of $T_{\text{A1}}$ transitional cell carcinomas of the urinary bladder by quantitative nuclear and proliferation related features

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Abstract

**Aim**—To analyse whether the mean nuclear area of the 10 largest nuclei (MNA-10), the mitotic activity index (MAI), and Ki-67 immunoquantitative features have additional value to discriminate different grades of $T_{\text{A1}}$ transitional cell carcinoma (TCC) of the urinary bladder.

**Materials/Methods**—One hundred and fifty of 200 consecutive cases (75%) showing interobserver agreement on duplicate blind grade assessment by independent pathologists were studied. Using random numbers, the 150 cases were divided into sets for learning (n = 75) and testing (n = 75). Single and multivariate analyses were applied to discriminate the different grades in the learning set. The multivariate classifier developed in this way was evaluated in the test set (n = 75).

**Results**—With the MNA-10 alone, using the classification $\text{MNA-10} < 80 \, \mu m^2 = \text{grade 1}$, $80 \, \mu m^2 < \text{MNA-10} < 130 \, \mu m^2 = \text{grade 2}$, $\text{MNA-10} > 130 \, \mu m^2 = \text{grade 3}$, 71% of all 150 cases were correctly classified (69% of grade 1 v grade 2 and 76% of grade 2 v grade 3). With multivariate analysis, the best discriminating features in the learning set (17 grade 1, 30 grade 2, and 28 grade 3) between grades 1 and 2 were MNA-10 and MAI, and between grades 2 and 3 MAI and Ki-67. With these features, 94% of grade 1 v grade 2 and 97% of grade 2 v grade 3 were correctly classified in the learning set (overall, 95% correct, none of the grade 3 cases misclassified). In the test set the classification results were similar. When the three grades were entered at the same time for discrimination, Ki-67 area % and MAI was the best discriminating combination, both in the sets for learning and testing. Overall correct classification results in the sets for learning and testing were slightly lower, but still 94% and 92%. Most importantly, none of the grade 3 cases was misclassified; the classification shifts all occurred between grades 1 and 2.

**Conclusions**—The combination of MNA-10, MAI, and Ki-67 gives much better discrimination between grades 1, 2, and 3 in $T_{\text{A1}}$ TCC of the urinary bladder than MNA-10 alone. The similarity of the classification results of the learning set and test set are encouraging and this quantitative pathological grading model should be applied in a prospective study.

Keywords: urinary bladder; bladder tumours; transitional cell carcinoma; Ki-67; morphometry; proliferation

The incidence of transitional cell carcinoma (TCC) of the urinary bladder is high. There are two main forms of this cancer, superficial and invasive, and the superficial form ($T_{\text{A1}}$) is the most frequent in the Western world. About 70% of $T_{\text{A1}}$ tumours recur after transurethral resection, both at the original site and new sites in the urinary bladder, and at recurrence approximately 20% show progression to a higher stage. In $T_{\text{A1}}$ tumours, grade is an important criterion for the prediction of outcome and also for therapeutic decision making. In many centres, patients with grade 1 and 2 $T_{\text{A1}}$ tumours are monitored on a regular basis (every three months for the first year), but grade 3 cancers are monitored even more intensively, and often also receive adjuvant local treatment. However, interobserver reproducibility of grading may be as low as 60–85%. Therefore, the need for objective grading criteria is high. Many groups have studied markers with the aim of improving the reproducibility of grading and the accuracy of the prediction of outcome. DNA ploidy is correlated with grade, but the assessment requires single cells to be prepared from the solid paraffin wax blocks. Mean nuclear area is easier to assess because standard histological sections can be used. The mean profile area of the 10 largest nuclei (MNA-10) found in a histological tumour section has proved to be valuable as an independent marker for grade and prognosis. In the study of Blomjous et al., DNA diploid or DNA aneuploid cases (measured by flow cytometry) had nearly the same survival as $\text{MNA-10} \leq 95 \, \mu m^2$ and $\text{MNA-10} > 95 \, \mu m^2$, respectively. Thus, it is tempting to speculate that high MNA-10 values are strongly predictive of DNA aneuploidy, which may be understandable from a cell biological point of view—many studies have shown that nuclear area strongly correlates with DNA content; thus, aneuploid cells often have larger nuclei. Indeed, using image cytometry highly aneuploid nuclei often have very large nuclei.

However, proliferation is also important in grading, and this is not reflected in the MNA-10 value. In many cancers, Ki-67 and the metaphase marker mitotic activity index (MAI) are widely accepted as proliferation associated markers. Although Pich and colleagues have studied Ki-67 among other parameters (argyrophilic nucleolar

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urothelial tumour cells could accurately be distinguished from other cells. Using these criteria, the sample point of the test grid could be Ki-67 positive, Ki-67 negative, or neither of the two. These last points were ignored. The Ki-67 area % was defined as \((\text{Ki-67 positive/} \text{Ki-67 positive + Ki-67 negative}) \times 100\).

Table 1  Descriptive statistics and probability of no difference (Mann-Whitney test) of the 150 grade 1, 2, and 3 transitional cell carcinomas of the urinary bladder

<table>
<thead>
<tr>
<th>Variable</th>
<th>Grade 1</th>
<th>P</th>
<th>Grade 2</th>
<th>P</th>
<th>Grade 3</th>
<th>P</th>
<th>Grade 4</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAI</td>
<td>34</td>
<td>34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MNA-10</td>
<td>82.0 (25.26)</td>
<td>&lt;0.0000</td>
<td>101.4 (21.30)</td>
<td>&lt;0.0000</td>
<td>208.0 (87.7)</td>
<td>&lt;0.0000</td>
<td>82.0 (25.26)</td>
<td></td>
</tr>
<tr>
<td>Ki-67 area %</td>
<td>4.6 (3.39)</td>
<td>&lt;0.0000</td>
<td>10.3 (5.93)</td>
<td>&lt;0.0000</td>
<td>29.3 (10.4)</td>
<td>&lt;0.0000</td>
<td>4.6 (3.39)</td>
<td></td>
</tr>
</tbody>
</table>

MAI, mitotic activity index; MNA-10, mean nuclear area of the 10 largest nuclei; P, probability of no difference.

Results
First we compared the MNA-10 measurements with the data of the original MNA-10 study of Blomjous et al.12 With the MNA-10 alone, using the classification MNA-10 < 80 \(\mu m^2\) = grade 1, 80 \(\mu m^2\) < MNA-10 < 130 \(\mu m^2\) = grade 2, MNA-10 > 130 \(\mu m^2\) = grade 3, 71% of all 150 cases were correctly classified (69% of grade 1 \(v\) grade 2 and 76% of grade 2 \(v\) grade 3). This confirmed the discriminative power of MNA-10 (fig 1). The results of the two different studies were remarkably similar. The larger variation in our study could be explained by the larger number of cases analysed. There was a trend for higher values of the quantitative features with higher grade. These differences were all highly significant between the grades (table 1). However, discrimination between the three grades was not perfect because there is considerable overlap. Figures 2 and 3 show the box plots of the other quantitative features studied and grade. As expected there was some discrimination but overlap was still large.

Selection by random numbers of cases for the learning and test sets for multivariate analysis resulted in the same number of different grades in each set: grade 1, \(n = 17\); grade 2, \(n = 30\); grade 3, \(n = 28\). In the learning set, the best discriminating combination of features between grades 1 and 2 was MNA-10 and MAI (fig 4). Note that there seems to be a dichotomy in the grade 2 cases: those that strongly resemble grade 1 cases and those that are at some distance from the grade 1 TCCs, with slightly higher values. The percentage of correctly classified cases in the test set with this combination was 94.7% (table 2). The best discriminating set of features between grades 2 and 3 was Ki-67 area % and MAI (fig 5). Here, the percentage of correctly classified cases in the test set was 97%. When the three grades were entered at the same time, Ki-67 area % and MAI was the best discriminating combination.
both in the set for learning and for testing (fig 6). Overall correct classification results with the three grades at the same time were slightly lower than with the separate analysis of two adjacent grades, but were still 94% in the learning set and 92% in the test set. Most importantly, none of the grade 3 cases was misclassified; the classification shifts all occurred between grades 1 and 2, and one grade 2 case was misclassified as grade 3.

Discussion

The purpose of our study on transitional cell carcinomas of the urinary bladder was to develop a reproducible method for grading that is simple and robust and thus may be used in daily patient care. We confirmed earlier findings that the mean of the largest 10 nuclear profiles subjectively detected at low magnification in a tissue section of TCCs can be used to distinguish three grades of differentiation, but with considerable overlap. At the start of our study, the question was whether discrimination between different grades would increase when quantitative features were combined. Indeed, the discriminating power of MNA-10 in combination with Ki-67 and MAI was found to be stronger than that of MNA-10 alone. A second important question was whether a multivariate combination of the quantitative features studied could be used routinely as a clinical grading tool. In this context, it was important that the classification results in the sets for learning and testing were similar. This shows that the classifying combination of features was robust. Our results are encouraging and this multivariate grading model should be applied in a prospective routine grading study. Such a study is essential to answer another important question: does the application of this multivariate quantitative model in routine practice increase the reproducibility of grading? Although the lack of observer reproducibility of grading is not an argument for leaving out quantitative features in clinical pathology, such a formal prospective reproducibility study of the quantitative grading model developed in our study is important.

In a previous study on Barrett’s oesophagus we found that the quantitative assessment of Ki-67 positivity method used here was the most reproducible and fastest out of several others analysed. This was in agreement with the reports of Fleege and colleagues and Gundersen et al. These studies demonstrated that point weighted sampling, as used in our study, is the most efficient estimator available, and thus the best theoretical and practical approach to assess volume percentages. However, although the best one available, the
method still slightly overestimates objects that are larger. Ki-67 positivity mainly occurs in late G1, and in early, mid, and late S phase cells. These nuclei may be larger than the Ki-67 negative G0 and early G1 nuclei. Thus, the method used may result in a slight positive bias or overestimation of Ki-67 positive nuclei, especially of the ones that are in very late S phase, which in general may be considerably larger. On the other hand, the Ki-67 positive nuclei of G1 and early S phase cells (which are nearly of the same size as G0, G1 Ki-67 negative nuclei) probably form most of the Ki-67 positive nuclei in many tumours. Theoretically, this reduces the degree of bias for large nuclei that are Ki-67 positive. Thus, it is unlikely that the theoretical overestimation of Ki-67 positive nuclei that might occur would have influenced the results significantly (see also the wide variations that occur in the Ki-67/MNA scatter plot).

Our study has re-confirmed the findings of Blomjous et al., putting the spotlight on MNA-10. There are two important questions that need to be answered. First, why is a simple tumour characteristic such as MNA-10 such a strong grade discriminator? Second, is the overall pattern of nuclear size, expressed in the mean nuclear area, not equally strong or stronger in TCCs than MNA-10? If so, the occurrence of cells with very large nuclear profile areas in histological tumour sections could be an irrelevant epiphenomenon. Although the prognostic studies of Blomjous and colleagues suggest that this is not the case and that MNA-10 is more important than the mean nuclear area of the overall population, more detailed investigations to answer this question are needed. Confirmation of the importance of MNA-10 might indicate that high MNA-10 values point to a clone of cells with a particularly aggressive nature, rather than being an incidental side effect. This immediately raises questions as to the molecular biological background of MNA-10. Many older publications in the 1960s and 1970s showed that nuclear area is strongly correlated with DNA content. Thus, aneuploid cells often have large nuclei. Indeed, with image cytometry, hypertetraploid highly aneuploid cells often have very large nuclei. Therefore, it is reasonable to hypothesise that high MNA-10 values may be related to aneuploidy or proliferation of G0 or G1 aneuploid nuclei. Moreover, with increasing grade MNA-10 also increases. This coincides with the frequent occurrence of aneuploidy in grade 3 tumours. Grade 3 TCCs have a greater likelihood than grade 1 and 2 TCCs of invading the lamina propria. Certain nuclei may be larger than the Ki-67 positive nuclei that are Ki-67 positive. Thus, it is unlikely that the theoretical overestimation of Ki-67 positive nuclei that might occur would have influenced the results significantly (see also the wide variation that occurs in the Ki-67/MNA scatter plot).

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