Cytological grading of breast cancer in Giemsa-stained fine needle aspiration smears

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INTRODUCTION

Therapy for breast cancer is based on several prognostic and predictive factors, which are usually obtained by postoperative histological, immunochemical or biochemical investigations of tumour tissue. However, some patients, e.g. patients with locally advanced disease, older patients with accompanying chronic diseases and patients who reject

Keywords: breast cancer, cytological nuclear grading, FNA breast cytology, histological grading, size of cell nuclei, anisonucleosis, nucleoli-containing nuclei, DNA-ploidy, S-phase fraction, tumour malignant potential, disease progression
surgery, are treated by other modalities. In such cases, some information necessary for treatment planning may be obtained by core biopsy. However, a less invasive fine needle aspiration of breast (BrFNA) can provide an adequate cell sample for morphological, immunocytochemical and flow-cytometrical tumour investigations as well.

In the mid 1970s, Wallgren and Zajicek showed that nuclear size, presence of nucleoli and the extent of cell clusters in a smear correlated well with disease progression. In the early 1980s, Moriquand created a cytological grade consisting of a semi-quantitative estimation of nuclear size, anisonucleosis, some chromatin features, nucleoli, cell clusters and the number of mitoses. Using this cytological grading system, breast cancer patients were classified into three prognostic groups. Many other attempts at cytological grading of breast cancer have been proposed since then, most of which are based on well established histological grading models (Table 1). However, the primary aim of the present study was to test a simple cytological nuclear grading system based on three basic nuclear features, size of nuclei, anisonucleosis and the proportion of nucleoli-containing nuclei, for its reliability in establishing the malignant potential of a tumour. In addition, we wanted to assess the impact of DNA-ploidy and S-phase fraction on histological grade (HG) prediction and their impact on the prediction of disease progression.

For nuclear feature assessment, we applied the results of one of our previous studies on cytological nuclear grading in which we analysed 85 Giemsa-stained BrFNA smears for eight nuclear features (size and shape of cell nuclei, chromatin density, chromatin structure and chromatin distribution, as well as the number, size and shape of nucleoli). All the nuclear features were evaluated for their importance in predicting the histological grade according to Elston's modification of Bloom-Richardson criteria (EBR). Three statistical analyses applied (cluster analysis, discriminant analysis, logistic regression analysis) pointed towards the same conclusion, namely, the reliability of discrimination between three histological grades by means of the nuclear features listed is poor (around 65%), while the reliability of discrimination between low histological grade and high histological grade is quite satisfactory (over 85%). Subsequently, we found that the most informative nuclear features were nuclear size, presence and shape of nucleoli and chromatin structure, along with chromatin density. However, in the present study, we decided to analyse the simplest cytological nuclear grading system possible which would be reliable enough, even in the hands of an inexperienced cytopathologist. Thus, we chose to analyse the three most easily evaluable morphological nuclear features: nuclear size, anisonucleosis and the proportion of nucleoli-containing nuclei (Table 2).

**MATERIALS AND METHODS**

Seventy-four patients with histologically-confirmed invasive ductal breast cancer, treated at the Institute of Oncology in Ljubljana between 1989 and 1993, were enrolled in the present study. The inclusion criteria were a preoperative cytopathological diagnosis of breast cancer followed by primary surgical treatment, which included mastectomy or partial breast resection with the dissection of axillary lymph nodes. Further inclusion criteria were histopathological grade assessment according to EBR criteria, and a follow-up record with the results of standard check-up used for breast cancer. Twenty patients had HGI tumours, 30 patients had HGII tumours and 26 patients had HGIII tumours.
<table>
<thead>
<tr>
<th>Year</th>
<th>No. of cases</th>
<th>Stain used</th>
<th>Features analysed</th>
<th>Nucleoli</th>
<th>Chromatin</th>
<th>Other</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thomas</td>
<td>1989</td>
<td>58</td>
<td>Di/C128-Quick</td>
<td>Clustering %</td>
<td>Size Shape</td>
<td>Presence -</td>
<td>HG</td>
</tr>
<tr>
<td>Hunt</td>
<td>1990</td>
<td>50</td>
<td>Giemsa</td>
<td>Clustering %</td>
<td>Size Shape</td>
<td>Presence - Necrosis</td>
<td>HG</td>
</tr>
<tr>
<td>Layfield</td>
<td>1991</td>
<td>26</td>
<td>Giemsa/PAP</td>
<td>Clustering</td>
<td>Bloom-Richardson’s nuclear grade</td>
<td>Mitoses</td>
<td>HG, DFS</td>
</tr>
<tr>
<td>Dabbs</td>
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<td>20</td>
<td>Diff-Quick, PAP</td>
<td>Clustering</td>
<td>Black’s nuclear grade</td>
<td>-</td>
<td>HG</td>
</tr>
<tr>
<td>Sneige</td>
<td>1992</td>
<td>100</td>
<td>PAP</td>
<td>-</td>
<td>Black’s nuclear grade</td>
<td>-</td>
<td>HG</td>
</tr>
<tr>
<td>Ciatto</td>
<td>1993</td>
<td>213</td>
<td>PAP</td>
<td>Moriquand cytological grading ref.2</td>
<td>-</td>
<td>-</td>
<td>DFS</td>
</tr>
<tr>
<td>Ducatman</td>
<td>1993</td>
<td>50</td>
<td>PAP/cytospin</td>
<td>%Single Shape</td>
<td>Presence Hyperchromasia Pattern</td>
<td>Mitoses Cellularity DNA-ploidy, SPF</td>
<td>HG</td>
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<td>Davey</td>
<td>1993</td>
<td>35</td>
<td>PAP/cytospin</td>
<td>-</td>
<td>Size Shape</td>
<td>Presence Structure DNA-ploidy, SPF</td>
<td>HG</td>
</tr>
<tr>
<td>DeGraaf</td>
<td>1994</td>
<td>68</td>
<td>Giemsa</td>
<td>-</td>
<td>Size Shape</td>
<td>Presence -</td>
<td>HG, DFS</td>
</tr>
<tr>
<td>Pleotis</td>
<td>1994</td>
<td>35</td>
<td>PAP</td>
<td>-</td>
<td>Bloom-Richardson’s nuclear grade</td>
<td>-</td>
<td>HG</td>
</tr>
<tr>
<td>Dabbs</td>
<td>1994</td>
<td>104</td>
<td>PAP/Diff-Quick</td>
<td>Tubules</td>
<td>Bloom-Richardson’s nuclear grade/Black’s nuclear grade</td>
<td>Mitoses</td>
<td>NG-HG*</td>
</tr>
<tr>
<td>Robinson</td>
<td>1994</td>
<td>281</td>
<td>PAP</td>
<td>%Single Margins</td>
<td>Presence Structure</td>
<td>Cell size Cell uniformity Mitoses, DNA-ploidy, SPF</td>
<td>HG</td>
</tr>
<tr>
<td>Cajulis</td>
<td>1994</td>
<td>100</td>
<td>PAP</td>
<td>-</td>
<td>Size Shape</td>
<td>Presence Clumping Mitoses, DNA-ploidy, SPF</td>
<td>HG</td>
</tr>
<tr>
<td>Idvall</td>
<td>1995</td>
<td>91</td>
<td>HE</td>
<td>-</td>
<td>Presence of large nuclei</td>
<td>-</td>
<td>Cytotype</td>
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<tr>
<td>Bozetti</td>
<td>1996</td>
<td>30</td>
<td>Giemsa</td>
<td>-</td>
<td>Size Shape</td>
<td>Presence DNA-ploidy</td>
<td>NG-HG*</td>
</tr>
<tr>
<td>Zoppi</td>
<td>1997</td>
<td>135</td>
<td>HE</td>
<td>-</td>
<td>Black’s nuclear grade</td>
<td>-</td>
<td>NG-HG*</td>
</tr>
</tbody>
</table>

HG = histological grade; DFS = disease free survival; *NG-HG = nuclear grade as a part of the EBR histological grading.
Fifty-nine patients also had the results of DNA-ploidy and S-phase fraction assessed preoperatively by flow cytometry. The median follow-up was 62 months.

On each Giemsa-stained smear, 200 cell nuclei were selected across the whole smear and scanned individually for their size and the number of nucleoli. To gain a random selection, the smears were scanned in 20 even intervals for 10 cells on each vertical line. The size of cell nuclei was evaluated in a semi-quantitative manner with one, two or three points, according to pre-set criteria, comparing cancer cell nuclei with the normal ductal cell nuclei (NDCN). Cell nuclei with a diameter of one and a half or less of the diameter of NDCN were assigned one point, those with a diameter between one and a half and two NDCN were assigned two points, and all the larger cell nuclei were assigned three points. Subsequently, the mean and standard deviations (SD) of nuclear size per smear were assessed. The interval between minimal and maximal SD of nuclear size of all smears was divided into thirds. Anisonucleosis of smears with SDs less than one-third of the minimum–maximum SD interval was assigned one point, anisonucleosis of smears with SDs larger than one-third but smaller than two-thirds of the minimum–maximum SD interval was assigned two points, and anisonucleosis of smears with larger SDs was assigned three points. In addition, the proportion of nucleoli-containing nuclei per 200 cells was assessed. Smears with 33% or fewer nucleoli-containing nuclei were assigned one point, smears with 33–66% were assigned two points and smears with more than 66% were assigned three points (Table 2). Finally, consistent with the EBR histological grading system, a three-grade cytological nuclear grade (CNG) was determined for each smear. Smears scoring 3–5 points were graded CNG I, those with 6 or 7 points were graded CNG II and smears with 8 or 9 points were graded CNG III.

Statistics

Pearson’s test was used for the evaluation of correlation of the three basic nuclear features, DNA-ploidy, S-phase fraction, HG and CNG. Logistic regression analysis was performed for the evaluation of potential predictive information of the three basic nuclear features, as well as that of CNG, DNA-ploidy and S-phase fraction, with regard to the EBR histological grade. Kaplan–Meier analysis with the log-rank test was performed for the evaluation of survival differences between patients with different cytological nuclear grades. Cox regression analysis was used for the evaluation of prognostic power of CNG, along with DNA-ploidy and S-phase fraction.

Table 2. The criteria for a semi-quantitative nuclear feature evaluation

<table>
<thead>
<tr>
<th>Nuclear feature</th>
<th>1 point</th>
<th>2 points</th>
<th>3 points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size*</td>
<td>&lt;1.68</td>
<td>1.68–2.33</td>
<td>&gt;2.33</td>
</tr>
<tr>
<td>Nucleoli (%)†</td>
<td>&lt;33</td>
<td>33–66</td>
<td>&gt;66</td>
</tr>
<tr>
<td>Anisonucleosis (SD)‡</td>
<td>&lt;0.34</td>
<td>0.34–0.54</td>
<td>&gt;0.54</td>
</tr>
</tbody>
</table>

* = Mean score of semiquantitative evaluation of size of 200 nuclei per smear. † = The proportion of nucleoli-containing nuclei per 200 nuclei.
RESULTS

Pearson’s correlation test showed a significant correlation between HG and size of nuclei, the proportion of nucleoli-containing nuclei, DNA-ploidy and S-phase fraction, and a nearly significant correlation with anisonucleosis. Furthermore, it showed a highly significant correlation between HG and CNG (Table 3).

Logistic regression analysis showed that, using the CNG system consisting of the three basic nuclear features, smears were correctly classified between the corresponding HG I and HG II in 73.47% ($P = 0.007, R = 0.28$). In the same way, smears were classified correctly between the corresponding HG II and HG III in 63.64% ($P = 0.013, R = 0.23$).

Following the results of our previous study mentioned above, in addition to clinical practice, we decided to create a two-grade CNG system, i.e., a CNG system consisting of low and high nuclear grades only. After joining both CNG II/III and the referential HG II/III into a single category, the smears were classified correctly between the corresponding low and high HGs in 79.73% ($P < 0.0000, R = 0.42$).

Logistic regression analysis was also employed for the analysis of the importance of the S-phase fraction and DNA-ploidy, in combination with CNG in the prediction of the corresponding HGs, but the results showed that neither S-phase fraction nor DNA-ploidy contributed to the reliability of the prediction of the corresponding HGs if CNG was considered at the same time.

Kaplan–Meier analysis showed a statistically significant difference in 5-year survival rate between the patients with CNG I and CNG II ($P = 0.031$), but no significant difference in

Table 3. The results of Pearson’s correlation test

<table>
<thead>
<tr>
<th>Pearson’s Correlation/Significance ($P$)</th>
<th>Size</th>
<th>Aniso</th>
<th>Nucl</th>
<th>S</th>
<th>DNA</th>
<th>HG</th>
<th>CNG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size$^1$</td>
<td>-0.175</td>
<td>0.281</td>
<td>0.310</td>
<td>0.489</td>
<td>0.424</td>
<td>0.430</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.136</td>
<td>0.014</td>
<td>0.017</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>aniso$^2$</td>
<td>-0.175</td>
<td>0.023</td>
<td>0.351</td>
<td>0.130</td>
<td>0.222</td>
<td>0.403</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.136</td>
<td>0.843</td>
<td>0.007</td>
<td>0.307</td>
<td>0.057</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>nucl$^3$</td>
<td>0.281</td>
<td>0.023</td>
<td>0.210</td>
<td>0.124</td>
<td>0.470</td>
<td>0.710</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.014</td>
<td>0.834</td>
<td>0.111</td>
<td>0.323</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>S$^4$</td>
<td>0.310</td>
<td>0.351</td>
<td>0.210</td>
<td>0.474</td>
<td>0.614</td>
<td>0.347</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.017</td>
<td>0.007</td>
<td>0.111</td>
<td>0.000</td>
<td>0.000</td>
<td>0.008</td>
<td></td>
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<tr>
<td>DNA$^5$</td>
<td>0.489</td>
<td>0.130</td>
<td>0.124</td>
<td>0.474</td>
<td>0.326</td>
<td>0.218</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>0.307</td>
<td>0.323</td>
<td>0.000</td>
<td>0.008</td>
<td>0.084</td>
<td></td>
</tr>
<tr>
<td>HG$^6$</td>
<td>0.424</td>
<td>0.222</td>
<td>0.470</td>
<td>0.614</td>
<td>0.326</td>
<td>0.539</td>
<td></td>
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<tr>
<td></td>
<td>0.000</td>
<td>0.057</td>
<td>0.000</td>
<td>0.000</td>
<td>0.008</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>CNG$^7$</td>
<td>0.430</td>
<td>0.403</td>
<td>0.710</td>
<td>0.347</td>
<td>0.218</td>
<td>0.539</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.008</td>
<td>0.084</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

1 = Size of cell nuclei; 2 = Anisonucleosis; 3 = No. of cell nuclei; 4 = S-phase fraction; 5 = DNA-ploidy; 6 = HG (histological grade); 7 = CNG (cytological nuclear grade).
survival of the patients with CNG II and CNG III ($P = 0.74$). After combining CNG II and CNG III into a single category, we observed a significant difference in 5-year survival rates between the patients with CNG I and CNG II + III, i.e., between the patients with low and high CNG ($P = 0.035$). These results are practically identical with the results of survival analysis of corresponding HGs (Figure 1a,b).

Figure 1. (a) Survival: a comparison between the three-grade and the two-grade CNG (cytological nuclear grade) system. Three-grade CNG: (––) grade III; (· · ·) grade II; (—) grade I. Two-grade CNG: (· · ·) high grade; (—) low grade. (b) Survival: a comparison between the three-grade and the two-grade HG (histological grade) system. Three-grade HG: (––) grade III; (· · ·) grade II; (—) grade I. Two-grade HG: (· · ·) high grade; (—) low grade.

Furthermore, Cox regression analysis, which included CNG, DNA-ploidy and S-phase fraction, showed weak prognostic power using the two-grade CNG system with regard to the overall survival ($P = 0.046$, \(RR = 2.95\) (95% confidence intervals = 1.015–8.56)), but no prognostic power in the use of DNA-ploidy and S-phase fraction. Neither of the three features examined showed prognostic power with regard to the disease-free survival.

**Figure 1.** Continued.
DISCUSSION

Our study shows that cytological nuclear grading in Giemsa-stained BrFNA smears of breast cancer, based on a semi-quantitative estimate of the three basic nuclear features, nuclear size, anisonucleosis and the proportion of nucleoli-containing nuclei, might present an alternative to histological grading. When testing this CNG on a sample of 74 breast cancer patients, we found a significant difference in survival between low-CNG and high-CNG breast cancer patients, but there was no difference in the survival rate between intermediate-CNG and high-CNG breast cancer patients. These results are in agreement with the corresponding survival analysis applying HG as an independent variable. Thus, a two-grade cytological/histological (CG/HG) grading system seems to bear the most relevant information for clinical practice. Our study also shows that DNA-ploidy and S-phase fraction correlate with HG as well as with the two-grade CNG. However, neither DNA-ploidy nor S-phase fraction in combination with CNG add any significant information to HG prediction, nor do DNA-ploidy and S-phase fraction in combination with CNG contribute significantly to the prediction of disease progression.

The subjective nature of all semi-quantitative grading systems is a strong argument for the opponents of semi-quantitative malignancy grading, although the later work on Elston’s modification of HG, one of the most widely used semi-quantitative histological grading systems, and the cytological nuclear grading reported by Sniege, show that well-defined criteria and good staff training result in optimal reproducibility of semi-quantitative grading systems. In that respect, we tried to analyse the semi-quantitative estimate of CNG in the most exact manner possible. This is why the cell nuclei were examined across the whole smear, the size of cell nuclei was estimated individually, anisonucleosis was calculated from the size of cell nuclei, and the nucleoli-containing nuclei were counted exactly.

The three basic nuclear features analysed in our study were chosen according to our routine experience that in Giemsa-stained smears, these nuclear features are easiest to evaluate. This is in agreement with the work of Schulte, who showed that in Giemsa-stained smears, morphological nuclear features, apart from chromatin pattern, can be well displayed and are more prominent than in wet fixed preparations. The results of our study are closely related to the results of many other authors who, in addition to the three basic nuclear features, took into consideration some other features as well (Table 1) Hunt, for example, analysed the combination of the three basic nuclear features plus the degree of cell clustering and the extent of necrosis. Consistent with our results, she found that the three basic nuclear features discriminate best between the corresponding HGs. She, too, showed an unreliable discrimination between the grades using a three-grade CG/HG method, but, in contrast to our study, she obtained a reliable correlation between CG and HG by combining low and intermediate CGs. In the same way, Thomas proposed a CNG model which was based on the three basic nuclear features plus cell clustering. His CNG model, as with ours, correlated reliably with HG by combining intermediate and high CNGs into a single category. He, however, found that the nucleoli-containing nuclei were evenly distributed among all three HGs, and found them irrelevant for any grading system. This conclusion is in sharp contradiction to our results, which show a highly significant correlation between the proportion of nucleoli-containing nuclei and HG (P < 0.000; R = 0.71). Furthermore, van Diest demonstrated a
statistically significant prognostic power for the nucleoli-containing nuclei present in a smear. His analysis showed that in the multivariate analysis, the number of nucleoli per 100 nuclei was the only independent prognostic factor among 42 morphometric nuclear and nucleolar variables.\textsuperscript{19}

Some authors report a reliable discrimination between the three CGs/HGs using additional sets of nuclear features. Bozzeti analysed a CNG consisting of the shape of nuclei and the three basic nuclear features. She found high concordance between CNG and HG (80\%) considering all three grades.\textsuperscript{20} Sniege, on the other hand, tested a modified Black’s nuclear grading system on 100 BrFNA smears, stained according to the Papanicolaou method, and found 90–96\% concordance between three CNGs and HGs. In addition, her group reported a 93–95\% intra-observer, and a 92–94\% inter-observer reproducibility rate, respectively. Consistent with the histological grading method, they investigated only the most anaplastic nuclei and did not make a general appraisal of the whole cell smear.\textsuperscript{9} Cajulis, however, tested a simplified two-grade modified Black’s nuclear grading system on 100 BrFNA Papanicolaou-stained smears stained and found 80–90\% concordance between high and low cytological and histological nuclear grades.\textsuperscript{12} Moreover, he tested a modified three-grade Black’s grading system (MB) vs. a simplified two-grade Black’s grading system (SB) in practice. He asked 15 private practice pathologists to grade the same set of 20 smears twice, using the MB system once and on a second occasion, the SB system. He found that two-thirds of the pathologists agreed in practically all cases (19 out of 20) when the SB grading system was used, while agreement between the participating pathologists was significantly lower when the three-grade MB grading system was used.\textsuperscript{21} This simple and efficient analysis clearly showed the practical value of two-grade grading based on an objective set of criteria, the same approach to the cytological nuclear grading we are trying to assess in the present study.

There are only a few analyses testing the prognostic power of a CG assessed on BrFNA smears. Ciatto considered cytological grading to be of no practical value, in spite of a clearly significant lower survival of patients with CG III tumours in comparison with the patients with CG I tumours, as shown in his study. He found that this significance was lost after including clinically assessed tumour size and the number of positive lymph nodes into a multivariate analysis.\textsuperscript{22} However, in the case of primary systemic treatment, the number of positive lymph nodes is usually not available, and the clinical assessment of tumour size is unreliable, especially when tumours are small or poorly delineated. In such cases, cytological grading might present a valuable prognostic factor. These facts are also in agreement with the results of our study, which shows that a two-grade CNG, based on the three basic nuclear features, reveals a significant difference in 5-year survival rate between the patients with low-grade and high-grade breast cancer.

In the search for objectivity in cytology, some authors have proposed automated, computer-powered methods of cytological smear analysis, based on morphometry, stereology, flow and image cytometry.\textsuperscript{25–27} However, these methods are not available in every cytopathology laboratory. As a rule, these are highly specialized methods which involve expensive equipment as well as time-consuming procedures for cell sample preparation and analysis, which can be performed only by specially trained staff. Nevertheless, in our study we also tested the potential prognostic information of DNA-ploidy and S-phase fraction measured by flow cytometry. We found that both DNA-ploidy and S-phase fraction correlated well with HG and CNG. However,
neither DNA-ploidy nor S-phase fraction in combination with CNG were significant in
the prediction of HG, nor were they significant in the prediction of disease progression.

In conclusion, in our study we showed that a CNG based on a semi-quantitative
estimation of the three basic nuclear features of nuclear size, anisonucleosis and the
proportion of nucleoli-containing nuclei, might present an alternative to the HG. With
cytological nuclear grading, as with histological grading, we can differentiate well between
the patients with high-malignancy and low-malignancy breast cancer. However, these
preliminary results, obtained by a clinico-pathological investigation of a small sample of
breast cancer patients, should be tested in a prospective study on a larger series of cases. In
addition, this was a pilot study, and indeed, a complex and a time-consuming one, far
from being simple and routinely applicable, but it clearly showed the significance of the
three basic nuclear features in cytological grading. For routine work, a simple semi-
quantitative evaluation of the three basic nuclear features applied on the whole smear
should be tested. We propose cell nuclei, in general, to be compared with any referential
object chosen, anisonucleosis relating to the extent of nuclear size variability to be
assessed, and the proportion of nucleoli-containing nuclei per 200 cells to be estimated.

According to our results, S-phase fraction and DNA-ploidy correlate well with HG.
However, neither of them contributes to the significance of the HG prediction, nor do they
contribute to the prediction of disease progression if CNG is considered at the same time.
In contrast to our results, some authors also showed the independent prognostic value of
S-phase fraction as well as that of DNA-ploidy. In view of these results, the combination
of DNA-ploidy, S-phase fraction and CNG should be tested again on a larger sample for
its potential prognostic power.

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