Prognostic Significance of DNA Ploidy Pattern and Nucleolar Organizer Regions (AgNOR) in Colorectal Carcinoma

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Aim. To investigate the prognostic significance of DNA ploidy and silver stained nucleolar organizer regions (AgNOR), as well as their relation to the histological grade and Dukes’ stages of colorectal carcinomas, and the relation of tumor cells proportion in the S-phase and Dukes’ stage, histologic grade, DNA ploidy, or AgNOR count.

Methods. DNA flow cytometric analysis and AgNOR were performed on 94 surgically removed colorectal carcinomas. The mean AgNOR count was calculated in 200 tumor cells for each case. Survival rates and tests for significance were evaluated using the log-rank test and Cox regression model.

Results. There were no significant correlations between the ploidy pattern, histological grade, and Dukes’ stage. Diploid tumors had a significantly lower AgNOR count (median 2.5, range 2.1-7.7) than aneuploid (median 6.2, range 2.0-7.9). Dukes’ C stage tumors exhibited higher AgNOR count than Dukes’ A or B stages. The proportion of tumor cells in S-phase did not correlate with any other parameter. Each of these parameters failed to show any correlation with survival. After dividing the tumors into those with high (>5) and low AgNOR count, no correlation was found in the latter group between AgNOR and any other studied parameters, whereas in the group with high AgNOR count correlations to Dukes’ stage, DNA ploidy, and histological grade were established.

Conclusions. The difference in survival between well, moderately, and poorly differentiated tumors were significant in the group with high AgNOR counts. Dukes’ C stage and aneuploid tumors had the worst prognosis.

Key words: cell cycle; colonic neoplasms; DNA, neoplasm; flow cytometry; nucleolus organizer region; prognosis, carcinoma; rectal neoplasms

Colorectal carcinomas are the most frequent tumors of the digestive tract (1). Mortality related to this disease is still high in spite of intensive screening and the use of methods that facilitate early diagnosis (colonoscopy, rectoscopy). Investigation of biological behavior of this tumor has been intensive and has resulted in the identification of new parameters which can be used in diagnosis, prognosis, and treatment choices. Two of them are DNA ploidy status (2) and silver stained nucleolar organizer regions (AgNOR) (3).

The relationship between DNA ploidy and many other factors, like tumor growth pattern, tumor differentiation, Dukes’ stage, and survival, has been evaluated, but the results have been contradictory. Some authors failed to establish any correlation between DNA content and survival (4-7), whereas others found lower survival rates for patients with aneuploid than for those with diploid tumors (8-11). Moreover, numerous studies have failed to reveal correlation between DNA ploidy and histological grade, Dukes’ stage or lymph node involvement (8,12-14), although some findings show that aneuploidy does correlate with histopathologic features (15).

The second quantitative method, which can be used as a malignancy marker, is the AgNOR method. Nucleolar organizer regions represent DNA segments associated with nonhistonic argyrophilic protein (16). The number of these regions
in the nucleus is directly proportional to protein synthesis and reflects the intensity of cell activity and cell proliferation (17,18). As the AgNOR count shows the intensity of cell proliferation, this method may be used in differentiating between benign and malignant tumors, as well as between tumors with high and low malignancy.

We analyzed the relationship between DNA ploidy and AgNOR count on one hand and histologic grade and Dukes’ stage on the other, as well as the relationship between the proportion of tumor cells in the S-phase and all these parameters. Each of these parameters was additionally related to the patient prognosis in order to determine whether DNA ploidy and AgNOR would be better indicators of tumor behavior.

Material and Methods

Histopathology

The study was performed on 94 surgically removed colorectal carcinomas. The clinico-pathological staging of tumors was performed according to Dukes’ classification (19). Hematoxylin and eosin stained sections were used for histological grading and adjacent samples were processed for flow cytometry as described below.

The differentiation of the tumors were classified as good, moderate, or poor (20). Well-differentiated adenocarcinomas exhibited complex or simple tubules, easily discerned nuclear polarity, and nuclei of uniform size. Moderately differentiated adenocarcinomas showed tubules, which were complex, simple, or slightly irregular and in which the nuclear polarity was barely discerned or lost. Poorly differentiated carcinomas were characterized by highly irregular glands or absence of glandular differentiation, and a loss of nuclear polarity.

DNA Flow Cytometric Analysis

The Hedley method for DNA analysis was used with some modification (21). Cell suspensions were prepared from paraffin embedded tissue. Three 30 mm sections were cut from each tissue block, deixed in xylene, and rehydrated through ethanol solutions (100%, 90%, 70%). After washing from each tissue block, dewaxed in xylene, and rehydrated shows the intensity of cell proliferation, this

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DNA Flow Cytometric Analysis

The Hedley method for DNA analysis was used with some modification (21). Cell suspensions were prepared from paraffin embedded tissue. Three 30 mm sections were cut from each tissue block, deixed in xylene, and rehydrated through ethanol solutions (100%, 90%, 70%). After washing in distilled water, 1.5 mL of 0.5% pepsin solution was added to each sample. The cell suspension was incubated at 37 °C for 45 min, filtered through a mesh, and centrifuged for 40 min at 1,650 rpm. The cells were resuspended in trypsin solution and incubated at 37 °C for 15 min. After filtration, the cells were again centrifuged for 40 min, and finally resuspended in phosphate buffered saline with 50 g/mL propidium iodide solution (PI) and 200 L RNAase, and incubated for 30 min at 37 °C. Flow cytometry on the FACScan device (Becton-Dickinson, Mountain View, CA, USA) was performed using CellFit Research software. The percentage of cells in G-01, S, or G-2+M phase were determined using the RFIT-model.

DNA Histogram Analysis

The ploidy status of tumors was determined by the amount of DNA, expressed as a DNA index (DI), i.e., the ratio between the peak channel number of the tumor sample in G-0/G-1 and the peak channel number of the diploid reference population in G-0/G-1. Unstimulated lymphocytes were used as reference standards. Tumor samples with a DI between 0.9-1.1 were marked as diploid, whereas those with a DI less than 0.9 or more than 1.1 were defined as aneuploid. One of the most important criteria for testing measurements is the coefficient of variation (CV). In this study, CV values below 10.0 were considered satisfactory. Finally, the S-phase fraction was used as a measure of the proliferative activity of the tumors.

Statistical Analysis

Statistical analysis included Fischer’s exact test of probability, Mann-Whitney test, ANOVA, Student’s t-test, Kruskal-Wallis test, and chi-square test. Survival rates and tests for significance were evaluated using the log-rank test and the Cox regression model.

Results

The mean age of 94 patients included in the study was 65 years (range 37-88 years). The patients were followed-up for 1-82 months, with an average of 39.9 months. The mortality rate was 55.3%. The median survival time was 43 months, and the three-year survival was 55.3 5.1% (Table 1).

Diploid tumors were more associated with lower AgNOR counts than aneuploid ones (Table 2). Nevertheless, there were no differences between the DNA ploidy pattern and well, moder-

Table 1. Clinicopathological parameters, DNA ploidy, AgNOR, and survival in 94 patients with colorectal carcinomas

<table>
<thead>
<tr>
<th>Tumor characteristics</th>
<th>n</th>
<th>Dead: No. (%)</th>
<th>Alive: No. (%)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>well</td>
<td>13</td>
<td>7 (53.9)</td>
<td>6 (46.2)</td>
<td></td>
</tr>
<tr>
<td>moderate</td>
<td>67</td>
<td>36 (53.7)</td>
<td>31 (46.3)</td>
<td>0.502</td>
</tr>
<tr>
<td>poor</td>
<td>14</td>
<td>9 (64.3)</td>
<td>5 (35.7)</td>
<td></td>
</tr>
<tr>
<td>Dukes’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>23</td>
<td>8 (34.8)</td>
<td>15 (65.3)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>59</td>
<td>37 (62.7)</td>
<td>22 (37.3)</td>
<td>0.079</td>
</tr>
<tr>
<td>C</td>
<td>12</td>
<td>7 (58.3)</td>
<td>5 (41.7)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>women</td>
<td>40</td>
<td>23 (57.5)</td>
<td>17 (42.5)</td>
<td>0.775</td>
</tr>
<tr>
<td>men</td>
<td>54</td>
<td>29 (53.7)</td>
<td>25 (46.3)</td>
<td></td>
</tr>
<tr>
<td>Ploidy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>diploid</td>
<td>45</td>
<td>25 (54.4)</td>
<td>21 (45.7)</td>
<td>0.594</td>
</tr>
<tr>
<td>aneuploid</td>
<td>48</td>
<td>27 (56.3)</td>
<td>21 (43.8)</td>
<td></td>
</tr>
<tr>
<td>S-phase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;7%</td>
<td>50</td>
<td>29 (58.0)</td>
<td>21 (42.0)</td>
<td>0.713</td>
</tr>
<tr>
<td>AgNOR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>64</td>
<td>34 (53.1)</td>
<td>30 (46.9)</td>
<td>0.414</td>
</tr>
<tr>
<td>&gt;5</td>
<td>30</td>
<td>18 (68.0)</td>
<td>12 (40.0)</td>
<td></td>
</tr>
</tbody>
</table>

*Log-rank test.
ately and poorly differentiated carcinoma, and no significant correlation between the DNA ploidy pattern and Dukes’ stage. Furthermore, there was no relationship between AgNOR counts and histological grade or Dukes’ stage. As the \( p \) value for Dukes’ stage was 0.094, relatively close to the marginally significant \( p \) value (0.05), we investigated the correlation of different Dukes’ stages and AgNOR number (A:B, A:C, B:C). There was no significant difference in the AgNOR count between Dukes’ A and B (\( p = 0.979 \)), but the difference was significant between Dukes’ A and C and Dukes’ B and C (Table 2).

Our study did not reveal any relationship between proportion of tumor cells in the S-phase and the other observed parameters (histological grade, Dukes’ stage, DNA ploidy, AgNOR count, age, or sex). Except for Dukes’ A and B stage, these factors did not influence the survival rate (Table 1).

In order to determine whether the death rates relative to all these parameters were related to AgNOR count, we divided tumors into two groups: those with low AgNOR counts (\( <5 \)) and those with high AgNOR counts (\( >5 \)). While no difference in survival relative to any of the parameters was found in the group with low AgNOR counts, the difference between well, moderately and poorly differentiated tumors was significant (\( p = 0.049 \)) in those with higher AgNOR counts. In addition, Dukes’ A-stage tumors were associated with the best prognosis, and Dukes’ C with the worst. Finally, these analyses confirmed that the prognosis was better in patients with diploid tumors and low AgNOR count, than in those with aneuploid tumors and a high AgNOR count (\( p = 0.032 \)) (Table 3).

### Discussion

The results of this study corroborate our previous findings that DNA and AgNOR pattern provide adequate basis for distinguishing two groups of tumors with different biological behavior (23) and confirm that these two parameters do have a prognostic significance. Since our initial examination failed to demonstrate any consistent correlation between the single parameters and survival except for the Dukes’ stage, we re-evaluated the prognostic value of these parameters after dividing tumors into those with high (\( >5 \)) and low (\( <5 \)) AgNOR counts. Again, no significant correlation was found in the group with low AgNOR counts. However, significant relationships were established between high AgNOR counts on one side, and histological grade, Dukes’ stage, and ploidy pattern on the other. The differences in survival were significant between well, moderately, and poorly differentiated tumors, with Dukes’ C and aneuploid tumors having the worst prognosis.

The results concerning the prognostic significance of DNA ploidy have been rather contradictory, and have been made even more complex by the findings (24,25) that most tumors have a heterogeneous DNA content. In one study, 30-60% of the examined cases were associated with more than one clone with a different DNA content (25). Nevertheless, it has been shown that DNA ploidy is a potential marker for predicting the response to chemotherapy (26) or an important indicator for the malignant transformation (27,28).

Our results with AgNOR demonstrated that this method alone was not a reliable prognostic factor. There was no evidence for the relationship between the survival rate and the AgNOR count, which is in accordance with other studies (29), although some of them did demonstrate that it correlated not only with the local progression but also with metastasing of colorectal carcinoma (30). We did not find a relationship between AgNOR count and S-phase either, which again is in line with some other reports that AgNOR values

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**Table 2.** AgNOR count in 94 colorectal carcinomas with respect to their DNA ploidy and Dukes’ stage

<table>
<thead>
<tr>
<th>AgNOR count (median, range)a</th>
<th>n</th>
<th>DNA index</th>
<th></th>
<th></th>
<th>Ploidy</th>
<th></th>
<th></th>
<th>S-phase</th>
<th></th>
<th></th>
<th>Sex</th>
<th></th>
<th></th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>diploid</td>
<td></td>
<td></td>
<td></td>
<td>aneuploid</td>
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<td></td>
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<td></td>
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<tr>
<td>AgNOR</td>
<td>46</td>
<td>2.5 (2.1–7.7)</td>
<td></td>
<td></td>
<td>48</td>
<td>6.2 (2.0–7.9)</td>
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<tr>
<td>Dukes</td>
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<tr>
<td>A</td>
<td>23</td>
<td>2.7 (2.0–7.6)</td>
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<tr>
<td>B</td>
<td>59</td>
<td>2.8 (2.0–7.7)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>12</td>
<td>5.4 (2.4–7.9)</td>
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</table>

aStatistics (Mann-Whitney test): AgNOR diploid vs. AgNOR aneuploid, \( p = 0.001 \); AgNOR Dukes’ A vs. AgNOR Dukes’ B, \( p = 0.039 \); AgNOR Dukes’ a vs. AgNOR Dukes’ C, \( p = 0.048 \); AgNOR Dukes’ B vs. AgNOR Dukes’ C, \( p = 0.036 \).
did not relate with Ki67 or PCNA expression (31,30). We did not find any relationship regarding the histological grade, although in other studies AgNOR count was found to be higher in moderately and poorly differentiated adenocarcinoma than in those which were well differentiated (32). Furthermore, AgNOR was reported to be a reliable for distinguishing colorectal adenoma from carcinoma (33).

To summarize, the evaluation of single parameters as possible prognostic factors for colorectal cancer proved to be of limited use. Moreover, the mutual inter-correlation of single parameters revealed very few significant relationships. An exception to this was the significant relationship between the DNA ploidy and the AgNOR count (low AgNOR correlating with diploid, and high AgNOR counts with aneuploid tumors). On the other hand, we found that if primary classification of tumors is performed according to AgNOR count, significant relationships could be established between prognosis and histological grade, Dukes' stage, and ploidy pattern. Thus, for a reliable prognosis of colorectal carcinoma, a set of parameters that relate to tumor behavior, rather than a single parameter, should be used.

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