DNA PLOIDY IN THE TREATMENT OF LARGE BOWEL ADENOCARCINOMA: A FOLLOW UP STUDY

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The aim of the study was to look for unfavourable prognostic features in colorectal cancer patients after their surgical treatment as well as to evaluate the prognostic value of cellular ploidy and proliferative activity.

Material and methods. A group of 71 colorectal cancer patients discharged from the hospital after surgical treatment in the years 1995-2000 was studied. The examined material was acquired from paraffin blocks of tumour segments. After histopathological verification, the tumour segments recovered from paraffin blocks were used for cytofluorometric analysis of cellular ploidy according to the Hedley method.

Results. Diploid tumours were found in 45 of 71 (63.4%) colorectal cancer patients. No significant relationship between DNA ploidy and sex, age, complications, inherited susceptibility to a disease, tumour magnitude, grade of histological malignancy, or grade of clinical progression was observed. After colorectal cancer surgery, the probability of five- and ten-year survival was 44.4% and 37.1%, respectively, for the patients with diploid tumours (DI<1.0), and 38.5% and 9%, respectively, for the patients with aneuploid tumours (DI>1.0). These differences were not statistically significant (p=0.120).

Conclusions. 1. Classical clinicopathologic factors are still the best prognostic criteria for the evaluation of the future results of colorectal cancer patients' surgical treatment. 2. Determination of cellular ploidy and proliferative activity of colonic adenocarcinoma cannot increase the ability to predict prognosis based on surgical treatment.

Key words: large bowel adenocarcinoma, DNA ploidy, proliferative activity of large bowel adenocarcinoma

Surgical treatment, with possible adjuvant therapy depending on the progression of disease, gives large bowel cancer patients the greatest possibility for recovery (1). However, as the evaluation of clinicopathologic criteria is often subjective and limited, data on prognosis are not objective, and if surgical treatment of large bowel cancer patients is unsuccessful, it is necessary to search for other unknown factors to identify the patients with a risk of a recurrence.

The purpose of this study is the following:
– to look for unfavourable prognostic features in large bowel cancer patients after their surgical treatment,
– to evaluate the prognostic value of cellular ploidy and proliferative activity of large bowel adenocarcinoma.
MATERIAL AND METHODS

A group of 71 large bowel cancer patients discharged from the hospital after surgical treatment in the years 1995-2000 were studied. These patients comprised 90% of all the patients operated on in that time in the hospital department due to large bowel cancers.

All data concerning the qualification of the patients for the operation, the operation procedure, the operative evaluation of disease progression, and the treatment immediately after the operation were obtained from the department’s archive documentation. The diagnostic procedure included colonoscopy or rectoscopy, an abdominal cavity ultrasonography in every patient, and abdominal cavity computer tomography in most of the patients. In 54 patients (76%), a colon contrast enema was performed. Chest X-rays in anteroposterior and lateral projections and electrocardiograms were performed in every patient. Before the planned operation, there was an attempt to establish a histopathologic diagnosis, which was possible in 47 (66.2%) cancer patients.

Retrospectively, on the basis of the operation protocols and histopathologic examination results from the resected tumour, lymph nodes, and clear margins, the progression grade of the disease was verified.

After discharge from the department, information about the patients, including their postoperative treatment, was obtained from the file documentation in the Surgical Outpatient Clinic. For the first five years, the patients were examined at least four times a year (every three months) and every six months thereafter. To confirm the date of death of a patient, the information obtained was verified in the Regional Unit of National Register Department of Communication and Information of the Ministry of Home Affairs (Terenowa Stacja Departamentu Rejestrów Państwowych Łączności i Informatyki Ministerstwa Spraw Wewnętrznych i Administracji), Katowice, and in the Centre of Analyses and Medical Statistics of the Silesian Public Health Centre (Ośrodek Analiz i Statystyki Medycznej Śląskiego Centrum Zdrowia Publicznego), Katowice.

Histopathologic analysis

The material to examine was obtained from paraffin blocks of tumour segments from 71 colorectal cancer patients under surgical treatment. The paraffin blocks were cut into microtome sections, about 5 µm thick each, and dyed with hematoxylin and eosin. Every histological specimen was examined independently by two pathomorphology workers. They confirmed the presence of large bowel adenocarcinoma in every patient and determined the grade of histological malignancy (G).

Preparation of the material for cytofluorometric analysis

After histopathological verification, the tumour segments were used for cytofluorometric analysis of cellular ploidy. All cytofluorometric examinations were made in the Department of Neoplasm Pathology of the Oncology Centre in Cracow (Zakład Patologii Nowotworów Krakowskiego Oddziału Centrum Onkologii).

To prepare a suspension of cellular nuclei from the paraffin-embedded tissues, a modification of the method described by Hedley et al. was applied (4).

In two or three 50 µm thick paraffin sections, the paraffin was removed in xylene. The sections were also hydrated in a series of alcohol solutions of decreasing concentration and etched in a 0.5% solution of pepsin (pH=1.5) at 37°C for 1 hour. The centrifugated suspension of cellular nuclei was incubated with propidium iodide (PI, Calbiochem) and then with ribonuclease (RNA – Sigma).

Dyed specimens were measured up to two hours after finishing the preparation. The specimens were collected and analysed in a flow cytometer FACSCalibur (Becton-Dickinson) equipped with an argon laser of 15 mW power with a wavelength equal to 488 nm.

At least 10 000 cellular nuclei were analysed at one time. Ploidy and proliferation were performed by a ModFit programme. Classification of DNA histograms in relation to ploidy was based on the criteria recommended by Shankey et al., presented at DNA Cytometry Consensus Conference in 1992 (5).

Histograms were described to be diploid when only one fluorescence signal was observed corresponding to G0/G1-phase cells. Any additional fluorescence signals or percentage of G2/M-phase cells above 20% qualified the histograms to be aneuploid. Aneuploidy degree was presented as DNA index (DI), i.e., ratio of fluorescence intensity for G0/G1-phase cells of abnormal population to fluorescence intensity for G0/G1-phase cells of a nor-
Diploid tumours were found in 45 (63.4%) of 71 large bowel cancer patients. In 13 patients (18.3%), remote metastases were found as well. Nine patients had metastases in the liver (seven with single lesions and two with multiple lesions), and four patients had pulmonary metastases. Other data are presented in tab. 1.

On admission to the hospital, the patients reported several types of complaints: bloody stool (81.7%), diarrhoea (77.5%) or constipation (76.1%), weakness (69%), abdominal pain (32.4%), and body mass loss (32.4%).

The relationship between cellular ploidy and different clinicopathologic factors in this group of colorectal carcinomas is presented in tab. 2.
The sex of the patients did not significantly influence the long-term results after surgical treatment (p=0.336).

The prognosis for the patients who were below 62 years of age at the time of carcinoma diagnosis was significantly more favourable (p=0.009). In this group, the probability of five-year survival was 53.3%, and that of ten-year survival was 44.8%. For patients over 62 years of age, the five- and ten-year survival probabilities were 34.2% and 9.8%, respectively.

In the group of patients under study, the lack of symptoms had a significant effect on more favourable prognosis. Probability of five-year survival was 83.3% in this group, and that of ten-year survival was 66.7%, in comparison to 15.6% and 11.1%, respectively, in the patients who complained of different symptoms (p<0.001) (fig. 1).

Large differences in survival probability in relation to tumour magnitude were found (p=0.023) (fig. 2). When the tumour was not bigger than 5 cm in largest diameter, the probability of five-year and ten-year survival was 58.3% and 26.7%, respectively, while these values were 25.7% and 22.7%, respectively, when the tumor diameter was equal to or bigger than 5 cm.

However, no statistically significant differences in the patients’ survival were observed between the groups with lower (T1+T2) and higher grades of T marker progression (p=0.138).

In the group of patients without metastases in the regional lymph nodes, the probability of five-year survival was 59.1%, and that of ten-year survival was 30.7%. However, in the case of metastases in the regional lymph nodes, the probability of ten-year survival was 14.8% in the patients after operation (p<0.001) (fig. 3).

When remote metastases were found at the same time as the primary tumour, the probability of survival was significantly lower (p=0.002). In the patients who did not have

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Table 2. Clinicophotologic factors and cellular ploidy of large bowel adenocarcinoma

<table>
<thead>
<tr>
<th>Factor</th>
<th>Ploidy pattern</th>
<th></th>
<th></th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>diploid</td>
<td>aneuploid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>19 (54.3%)</td>
<td>16 (45.7%)</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>26 (72.2%)</td>
<td>10 (27.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 62</td>
<td>21 (70%)</td>
<td>9 (30%)</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>≥ 62</td>
<td>24 (58.5%)</td>
<td>17 (41.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trouble</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Yes</td>
<td>37 (64.9%)</td>
<td>20 (35.1%)</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>8 (57.1%)</td>
<td>6 (42.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neoplasm in family</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>22 (61.1%)</td>
<td>14 (38.9%)</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>23 (65.7%)</td>
<td>12 (34.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumour magnitude</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5</td>
<td>22 (61.1%)</td>
<td>14 (38.9%)</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>≥ 5</td>
<td>23 (65.7%)</td>
<td>12 (34.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T marker</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1+T2</td>
<td>24 (63.2%)</td>
<td>14 (36.8%)</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>T3+T4</td>
<td>21 (63.6%)</td>
<td>12 (36.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N marker</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>26 (59.1%)</td>
<td>18 (40.9%)</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>N+</td>
<td>19 (70.4%)</td>
<td>8 (29.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M marker</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>37 (63.8%)</td>
<td>21 (36.2%)</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>8 (61.5%)</td>
<td>5 (38.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade G</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>19 (67.9%)</td>
<td>9 (32.1%)</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>G2+G3</td>
<td>26 (60.5%)</td>
<td>17 (39.5%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
remote metastases, the probability of five-year survival was 48.3%, and that of ten-year survival was 27.4%, while in the patients with remote metastases, the probabilities were 15.4% and 7.7%, respectively.

Grade of histological malignancy (G) of colorectal adenocarcinomas (p<0.001) had a significant effect on differences in the patients’ survival probabilities (fig. 4). When the grade of malignancy (G1) was low, the probability of five-year survival was 71.4%, and that of ten-year survival was 50.8%, while these values were 23.3% and 8.1%, respectively, in the case of higher grades of histological malignancy (G2+G3).

After operation due to colorectal cancer, the probabilities of five- and ten-year survival were 44.4% and 37.1%, respectively, for the patients with diploid tumours (DI=1.0), and 38.5% and 9.0%, respectively, for the patients with aneuploid tumours (DI>1.0). These differences were not statistically significant (p=0.120) (fig. 5).

When the median of the percentage S-phase cells was considered as a boundary value, the comparison of patients’ survival probability was not statistically significant (p=0.157) (fig. 6). The probabilities of five- and ten-year survival were 48.3% and 44.8%, respectively, if the percentage of S-phase cells was lower than the median (9.25%), and 37.5% and 25%, respectively, when this value was equal or higher than the median.

### Multivariate analysis

To have a satisfactory evaluation of a prognostic value of proliferative activity markers, a multivariate analysis of total survival time of colorectal cancer patients was carried out with the use of Cox’s method of proportional risk regression, taking classical clinicopathologic factors into account. The analysis results allow the determination of the following independent unfavourable prognostic features (tab. 3):

![Fig. 3. Effect of metastases in regional lymph nodes on remote results of surgical treatment of large bowel cancer patients](image)

![Fig. 4. Effect of histologic malignancy grade (G) on remote results of surgical treatment of large bowel cancer patients](image)

![Fig. 5. Effect of cellular ploidy on remote results of surgical treatment of large bowel cancer patients](image)

![Fig. 6. Effect of S-phase percentage on remote results of surgical treatment of large bowel cancer patients](image)
DNA ploidy in the treatment of large bowel adenocarcinoma: a follow up study

Table 3. Analysis of risk factors for death due to large bowel cancer (Cox’s proportional hazard regression method)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Value Beta</th>
<th>Standard Error Beta</th>
<th>Value t</th>
<th>Exponent Beta</th>
<th>Statistic Value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>-0.044</td>
<td>0.333</td>
<td>-0.131</td>
<td>0.957</td>
<td>0.917</td>
<td>0.886</td>
</tr>
<tr>
<td>Age</td>
<td>0.052</td>
<td>0.116</td>
<td>3.237</td>
<td>1.053</td>
<td>10.480</td>
<td>0.001</td>
</tr>
<tr>
<td>Neoplasm in family</td>
<td>0.101</td>
<td>0.233</td>
<td>0.432</td>
<td>1.106</td>
<td>0.186</td>
<td>0.666</td>
</tr>
<tr>
<td>Tumour location</td>
<td>0.090</td>
<td>0.282</td>
<td>0.319</td>
<td>1.094</td>
<td>0.102</td>
<td>0.749</td>
</tr>
<tr>
<td>Tumour magnitude</td>
<td>-0.498</td>
<td>0.460</td>
<td>-1.084</td>
<td>0.608</td>
<td>1.175</td>
<td>0.278</td>
</tr>
<tr>
<td>Marker T</td>
<td>0.113</td>
<td>0.261</td>
<td>0.431</td>
<td>1.119</td>
<td>0.186</td>
<td>0.667</td>
</tr>
<tr>
<td>Marker N</td>
<td>0.811</td>
<td>0.302</td>
<td>2.690</td>
<td>2.251</td>
<td>7.238</td>
<td>0.007</td>
</tr>
<tr>
<td>Marker M</td>
<td>1.281</td>
<td>0.452</td>
<td>2.836</td>
<td>3.601</td>
<td>8.045</td>
<td>0.005</td>
</tr>
<tr>
<td>Histologic malignancy grade (G)</td>
<td>0.912</td>
<td>0.445</td>
<td>2.048</td>
<td>2.490</td>
<td>4.196</td>
<td>0.041</td>
</tr>
<tr>
<td>Ploidy pattern</td>
<td>1.263</td>
<td>0.662</td>
<td>1.907</td>
<td>3.535</td>
<td>3.638</td>
<td>0.056</td>
</tr>
<tr>
<td>S-phase %</td>
<td>0.003</td>
<td>0.014</td>
<td>0.233</td>
<td>1.003</td>
<td>0.054</td>
<td>0.816</td>
</tr>
</tbody>
</table>

- old age of the patients at the time of diagnosis (p=0.001),
- metastases in regional lymph nodes (p=0.007),
- remote metastases (p=0.005),
- high grade of histological malignancy (G) (p=0.041).

DISCUSSION

It is still believed that extensiveness of malignant disease and possibility of a radical operation are the most important prognostic features to evaluate long-term results in the treatment of colorectal cancer patients (1, 2, 3). However, cancer may develop for many years before it is clinically diagnosed and may cause micrometastases. Thus, colorectal carcinoma is in fact a systematic disease at the moment of a diagnosis (6).

A review of the literature shows that the character of cellular ploidy determined by flow cytofluorometry (FCM) and the grade of carcinoma proliferation (SPF or IP) may be a helpful supplementary data for the evaluation of clinicopathologic factors (2). They can also help in evaluating the cytostatic resistance of the neoplasm, tumour invasiveness, and potential to give metastases (7).

Many studies report a correlation between cellular ploidy pattern and long-term results in the treatment of colorectal cancer patients (2, 3, 8). Some authors even conclude that aneuploidy is an independent unfavourable prognostic feature (8-11).

Kokal (12), who studied 147 patients, claims that the pattern of colorectal neoplasm ploidy is a prognostic feature that is more vital than the grade of clinicopathologic progression according to Duke’s classification. Also, Takanishi (13) proved that DNA ploidy was an independent prognostic feature, but only among 210 patients with grade B according to Duke’s classification. However, the results of other authors’ my studies as well do not confirm these relations (14,15).

Chen (3), basing on a study of 666 colorectal cancer patients, did not find a significant relationship between cellular ploidy and SPF and long-term treatment results. Also Enker (16), Melamed (14), and Finan (17) were not able to prove better survival if the tumours were diploid in groups of 176, 33, and 46 patients, respectively. Boettger (18) studied 137 colorectal cancer patients and showed a relationship between prognosis and the tumour location, depth of infiltration into neighbouring structures, metastases into regional lymph nodes, and grade of histological malignancy, but the prognosis was independent of cellular ploidy. Tonouchi (9) did not observe a better prognosis for the patients with diploid tumours, either, and according to his study, penetration of the tumour into the colon wall was the most significant prognostic feature. A similar conclusion was drawn by Olson (19), who wrote that recurrence following curative resection depended on metastasis into regional lymph nodes and infiltration into neighbouring tissues, excluding the serosa. Our results confirm that metastases in regional lymph nodes and grade of histological malignancy in colorectal cancer patients after operation are the strongest prognostic features.

Sasaki (20) showed a correlation between more frequent aneuploidy and higher grade of...
histological malignancy in the group of colorectal carcinomas. However, our results do not confirm this correlation.

In the group of 71 patients in this study, 36.6% of neoplasms had an aneuploid pattern. These results are in agreement with those obtained by other authors, who demonstrated that the average percentage of aneuploid tumours is in the range of 35 to 89% (21, 22).

Scott (11) showed that there was no correlation between DNA ploidy and grade of clinical progression in 264 colorectal cancer patients. Similar observations were made by Kokal (12) in a group of 147 patients and Tono-uchi (9) in 75 patients. However, Pinto (22), showed in 61 patients that DNA ploidy correlated with histopathologic indicators. He demonstrated that there was a statistically significant relationship between DNA ploidy and the grade of clinicopathologic progression in colorectal carcinomas. He observed that the more advanced the grade, the more frequently tumours had an aneuploid pattern. In grade I, about 75% of colorectal carcinomas had a diploid pattern, but in grade IV, all carcinomas were aneuploid. Dean and Vernava (21) compared the results from 16 works and observed a slight tendency towards more frequent aneuploidy in more advanced tumours. This suggests that aneuploidy may be faster together with tumour progression (8, 12, 22).

The relationship between aneuploidy and higher grade of histological malignancy is also a subject of controversy. Dean and Vernava (21) did not observe such relationships, while Ba- zan (10) showed a statistically significant increase in aneuploidy frequency in colorectal carcinomas with higher grade of histological malignancy.

Differences in DNA within the same neoplasm can be observed in a wide range (5-49%) of solid tumours (9, 15, 18). It can be quite often observed that various types of cells in relation to DNA ploidy pattern may co-exist within one tumour. Due to such heterogeneity, the cellular ploidy pattern seems to be dependent on the place where tumour material is extracted. Some authors claim that DNA analysis from one segment only is not sufficient (20, 23, 24). Rognum (15) observed that the more segments of the tumour that were examined, the more frequently aneuploid tumours were recognized.

Sugai (24) showed that among in 146 of 164 (89%) cases of colorectal carcinoma, at least one of six segments was aneuploid. He concluded that if a single segment is examined, aneuploid clones may be masked by assuming a CV that is too high, which results in false interpretation and qualifying such a clone to be diploid. Sasaki (10) confirmed this opinion, as he recognized multiploidy in 68% peaks G1/G0 at CV below 3%. He also showed that a larger number of biopsies makes cellular subpopulations more recognizable within the same tumour by 37% (20).

It seems obvious that depth of invasion is one of the most important prognostic features in patients undergoing surgery due to colorectal carcinomas (22).

Although the determination of S-phase fraction as a proliferation index is clinically useful, in the evaluation of tumour progression it is less useful than DNA ploidy determination (3, 5).

It has been previously shown that in colorectal carcinoma the percentage of S-phase cells and the grade of TNM progression are the most important independent prognostic features (16, 25). However, our studies, similarly to others, did not show such a relationship (3, 26-30).

It was shown in many studies that aneuploid colorectal carcinomas had higher SPF than diploid tumours, and this has been confirmed by our studies (25, 29).

In our studies we also verified the correlation between the percentage of S-phase cells and clinicopathologic characteristics such as the magnitude of the neoplasm, metastases in the regional lymph nodes, and remote metastases. However, we did not find any relation.

CONCLUSIONS

1. Classical clinicopathologic factors are still the best prognostic criteria for the evaluation of remote results of large bowel cancer patients’ surgical treatment.
2. Determination of cellular ploidy and proliferative activity of large bowel adenocarcinoma cannot increase the ability to predict prognosis based on surgical treatment.
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REFERENCES


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