The diagnostic and prognostic value of genetic aberrations in resectable distal bile duct cancer
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Chapter 4

DNA Quantitation of Distal Bile Duct Carcinoma Measured by Image and Flow Cytometry

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MATERIAL AND METHODS

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Chapter 4

ABSTRACT

Background. Flow cytometry (FCM) and image cytometry (ICM) have provided the basis for much of our knowledge of the prognostic value of DNA-ploidy in a wide variety of tumours. Theoretically, FCM has a strong statistical basis whereas ICM is more specific in identifying smaller abnormal cell populations. Distal bile duct carcinomas (DBDCs) often contain dense non neoplastic stroma, and ICM could therefore be more desirable to measure DNA-ploidy. We applied FCM and ICM to resected DBDCs to evaluate discrepancies between both methods, the ploidy incidence, and the relation between DNA-ploidies and survival in these tumours.

Methods. Forty-four archival tumour samples of patients with DBDC, who underwent subtotal pancreatoduodenectomy from 1985 to 1996, were examined for DNA-ploidy, using FCM and ICM.

Results. Overall 59% (26/44) of the tumours were aneuploid by at least one of the two techniques. We detected more cases of aneuploidy with ICM than FCM in formalin-fixed, paraffin-embedded DBDCs, 62% (21/34) versus 33% (13/40), respectively. When results could be compared, a moderate strength of agreement (Kappa=0.45) was demonstrated. Twelve aneuploid tumours were detected by ICM but not by FCM. One near-diploid tumour (DNA index of 1.09) was recognised by flow but not by image. No correlation was found between DNA-ploidy by FCM, ICM or combined FCM-ICM and survival time (p=0.80, p=0.35, and p=0.54, respectively).

Conclusion. We have shown in a comparative study that approximately 59% of DNA histograms contained aneuploid cell populations. Although ICM as compared to FCM is more sensitive to assess the ploidy status of DBDC, both methods were complementary. Most discrepancy between FCM and ICM were due to the dilution of aneuploid populations by non neoplastic diploid cells. DNA-ploidy assessment in DBDC, unfortunately, did not offer the possibility to improve the ability to predict survival.

INTRODUCTION

There is considerable evidence to suggest that most human neoplasms contain detectable chromosome abnormalities. The development of two different cytometry techniques, flow and image, have facilitated the measurement of DNA content in dissociated cells of both fresh and archival material. Flow cytometry (FCM) requires a suspension of single nuclei, stained with a DNA-specific fluorescent dye, which makes it possible to quantitate the DNA content of a large population rapidly (>10,000 nuclei) by measuring their fluorescence. Although FCM is an excellent tool when moderate to large numbers of aneuploid cells are present, this method requires a quantitative DNA abnormality of at least 5% of the total DNA content for detection. Image cytometry (ICM) also analyses the DNA content of nuclei, the difference being the use of Feulgen-stained nuclei that have been fixed onto microscope slides. One of the obvious benefits of an image system
is its ability to visualise the nuclei to be evaluated, allowing the operator to select intact tumour nuclei based on morphology. As a result, specimens containing smaller numbers of malignant cells can be directly analysed and the operator has a very clear understanding of the link between the DNA histogram measured and the tumour sample.

The results of both techniques can usually be presented in an easily interpretable histogram. An aneuploid histogram displays a visually identifiable additional G0/G1 peak which corresponds to a DNA content different from the normal (2N) diploid standard. In a previous study, aneuploidy served as a marker for malignancy, although it can not always be equated with malignancy because some benign tumours may exhibit chromosomal abnormalities.

In several types of human malignant tumours, for example pancreatic carcinoma, there is considerable evidence to suggest that DNA content correlates with long-term prognosis. Literature, however, conflicts rather about the diagnostic value of ploidy status. Carcinomas arising from the head of the pancreas are considered periampullary carcinomas, which also include ampullary carcinoma and distal bile duct carcinoma (DBDC). In comparison to other tumours, aneuploidy in DBDC remains incompletely characterized. In a previous study we analysed DNA-ploidy by FCM in 35 patients with DBDC, revealing an aneuploidy frequency of 35%. No correlation between DNA-ploidy and prognosis could be found.

Although comparative studies using FCM and ICM for DNA content analysis of fresh tissue showed high concordance rates, most of the discordant cases showed aneuploid peaks only on ICM and not on FCM. Based on these findings, we extended our series and used ICM to screen archival DBDC specimens for previously undetected small (<5%) aneuploid populations. The main advantage of using paraffin-embedded tissue is that a relatively large series of patients with this rare type of tumour can be studied. To evaluate the incidence and prognostic value of aneuploidy in DBDC in more detail, and to evaluate any discrepancies between techniques, we performed FCM and ICM of 44 tumour samples.

**MATERIAL AND METHODS**

**Patients**
Formalin-fixed, paraffin embedded specimens from 44 patients who underwent subtotal pancreateoduodenectomy (Whipple procedure) for cancer of the distal bile duct, were amenable to study. The Whipple procedure was performed at the Academic Medical Centre between 1985 and 1996. All resections were carried out by a group of surgeons experienced in hepatobiliary surgery, and all the operations were intended to be curative. Of these 44 patients, 35 were male and 9 female, with a mean age of 60 years (range 37-77 years). Clinical and histopathological re-evaluation confirmed that all tumours were DBDCs located in the intrapancreatic portion of the common bile duct.
Single cell preparation for flow and image cytometry

Of the 44 tissue blocks, 4 μm sections were cut for hematoxylin and eosin staining. The densest area of tumour in at least 3 tissue blocks per resected specimen was selected and 1 to 3 sections of 50 μm thickness were cut for DNA measurements. The 50 μm sections were microdissected to exclude non-tumour (stromal) tissue. A modification of the basic method of cell preparation as described by Hedley and co-workers \(^\text{18}\) was employed. The sections were deparaffinized and then disintegrated for 30 minutes at 37°C in 1 ml of 0.5% Trypsin (Sigma 8128) in Tris HCL and the pH was adjusted to 7.6. After washing in phosphate-buffered saline (PBS), the specimens were dissociated for image and flow cytometry.

Image Cytometry

The specimens were centrifugated for 5 minutes, and smears were prepared from the sediment. In addition, the slides were air dried and stained by the Feulgen method. Briefly, the slides were placed in 96% ethanol for 30 minutes and then in 5N HCL at 27°C for 30 minutes, washed twice with PBS and stained with fresh Schiff reagents for 45 minutes and washed in running tap water for 15 minutes.

The slides were washed and then ICM measurements were performed with a microscope TV-system consisting of an Axioplan microscope (Zeiss, Oberkochen, Germany) equipped with a 20x plan apochromat, numerical aperture 0.60 and a projective magnification of 1.6x. Images were recorded by a XC-77-CE CCD (Sony, Köln, Germany) black and white camera with a CCD-cell size of 11 x 11 μm. The section was moved with an automatic scanning stage (Mährhäuser, Wetzlar, Germany) with a step size of 0.25 μm and an autofocus device (Zeiss, Oberkochen, Germany) operating on the TV-signal took care of the focussing (step size 0.025 μm). Image processing was performed on a Sparc 10 model 30 workstation (Sun Microsystems Inc., Mountain View (CA), United States) running under the UNIX (SunOS, Sun Microsystems Inc., Mountain View (CA), United States) operating system with a colour monitor at a spatial resolution of 1152x900 in 8 bit. The automatic DNA image cytometer was developed and evaluated within the Pathology Image Processing Environment which is based upon the multi-level interactive image processing environment SCIL-Image (DIFA measuring systems, Breda, The Netherlands). The system follows the guidelines of the consensus report of the European Society for Analytical Cellular Pathology \(^\text{19}\). The images were linearly corrected for shading with two empty images, one illuminated and one dark-current image \(^\text{20}\). The corrected grey values thus provide a measure for the local optical density. Segmentation was done fully automatically based on the algorithm described by Vossepoel et al. \(^\text{21}\), and a filter to remove debris and aggregate was active during measurement. At least 1000 cells were measured automatically, and results were inspected afterwards visually to remove remaining debris and aggregate from the data set, relocating cells if necessary. In the histograms, the 2c peak was identified interactively, after which the histogram was scaled up to 10c with a fixed number of 256 bins in order to obtain standardized histograms for all cases and exported in ASCII format. Like others \(^\text{22}\), we regarded
samples with coefficient of variation (CV) values above 10 as unacceptable. When large amounts of necrotic debris were present on the slides this was also considered non-diagnostic. An average of 857 (range 774-992) cells were measured per slide. Mean CV of the G0/G1 peak in diploid histograms measured by ICM was 7.3% (range 3.2-10.0).

**Flow cytometry**
Nuclear DNA was stained with propidium iodide by the detergent/trypsin method described by Vindelov et al.\textsuperscript{23}. Before analysis, all samples were filtered through a 40 μm nylon mesh to remove any residual tissue fragments. Nuclear DNA content of 10,000 to 20,000 nuclei per sample were collected and analysed with the use of a Becton Dickinson FACScan\textsuperscript{°} (Mountainview, CA). Flow samples with CV values exceeding 8% were rejected from this study. Mean CV of the G0/G1 peak in diploid histograms measured by FCM was 5.6% (range 2.7-8.0).

**Interpretation of DNA histograms**
In spite of microdissection, samples will contain inflammatory and stromal cell nuclei, providing an optimum internal diploid standard for the determination of the ploidy pattern\textsuperscript{24,25}. The channel number of the first peak in the histogram was defined as diploid G0/G1. The DNA index (DI) was defined as the ratio of the mode (or mean) of the relative DNA content of the G0/G1 cells of the sample divided by the mode (or mean) of the relative DNA measurement of the diploid G0/G1 reference cells. Aneuploidy was defined as one or more distinct separate peaks compared with the G0/G1 and G2M peaks of the diploid cell population. A histogram was defined as tetraploidy when the G2M peak of the first cell cycle (diploid) contained more than 10% of the total number of cells of that cell cycle. Histogram classification was performed without previous knowledge of the clinico-pathologic or survival data.

**Statistical Analysis**
Survival curves were performed using the Kaplan-Meier method, and univariate survival comparisons were made using the log-rank test (SPSS 6.0 statistical software). P<0.05 was regarded as statistically significant. The Kappa statistic was used as a measure of agreement between FCM and ICM corrected for chance agreement\textsuperscript{26}. We interpreted Kappa <0.20 as poor, 0.21> Kappa <0.40 as fair, 0.41> Kappa <0.61 as moderate, 0.61> Kappa <0.80 as good, and 0.81> Kappa <1.00 as very good strength of agreement.

**RESULTS**
All cases were interpretable by at least one of the two techniques, revealing an overall aneuploidy incidence of 59% (26/44). As can be seen in Table 1, in 4 out of 44 (9%) carcinomas analysed, flow cytometric assessment was impossible or unreliable despite repeated measurements, because the CV of the diploid peak was exceeding 8% without...
an aneuploid peak being present. FCM demonstrated a definite aneuploid peak in 13 (33%) of the remaining 40 cases. Ten (23%) image cases had to be excluded from the study due to the inability to obtain good DNA histograms from the tumours. ICM detected 62% (21/34) aneuploid populations in the remaining 34 cases.

Table 1. Summary of DNA analysis by flow cytometry (FCM) and image cytometry (ICM).

<table>
<thead>
<tr>
<th>Method</th>
<th>No. of Cases Examined</th>
<th>No. of Cases Uninterpretable</th>
<th>DNA Ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Diploid</td>
</tr>
<tr>
<td>FCM</td>
<td>44</td>
<td>4</td>
<td>27 (67%)</td>
</tr>
<tr>
<td>ICM</td>
<td>44</td>
<td>10</td>
<td>13 (38%)</td>
</tr>
</tbody>
</table>

Concordance between FCM and ICM
Thirty cases were interpretable by both, FCM and ICM, and comparison of the DNA results showed a 56% (17/30) concordance. The Kappa statistic for the scores was 0.45; this suggests moderate agreement between both methods. Diploidy was confirmed in 30% (9/30) and aneuploidy in 27% (8/30) of these tumours. The correlation of the DNA indices of aneuploid populations encountered by both techniques was striking. In only one case there was a discrepancy between the DI found by FCM and ICM, 1.80 and 1.15, respectively.

Table 2. Aneuploid populations detected by image cytometry (ICM) but not by flow cytometry (FCM).

<table>
<thead>
<tr>
<th>Case No</th>
<th>ICM / DI*</th>
<th>FCM / CV†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>aneuploid / 1.35</td>
<td>diploid / 5.20%</td>
</tr>
<tr>
<td>5</td>
<td>aneuploid / 1.22</td>
<td>diploid / 5.60%</td>
</tr>
<tr>
<td>6</td>
<td>aneuploid / 1.26</td>
<td>diploid / 3.80%</td>
</tr>
<tr>
<td>10</td>
<td>aneuploid / 1.68</td>
<td>diploid / 6.50%</td>
</tr>
<tr>
<td>16</td>
<td>aneuploid / 1.40</td>
<td>diploid / 4.70%</td>
</tr>
<tr>
<td>17</td>
<td>aneuploid / 1.70</td>
<td>diploid / 5.70%</td>
</tr>
<tr>
<td>20</td>
<td>aneuploid / 1.81</td>
<td>diploid / 3.80%</td>
</tr>
<tr>
<td>22</td>
<td>aneuploid / 1.28</td>
<td>diploid / 6.90%</td>
</tr>
<tr>
<td>25</td>
<td>aneuploid / H</td>
<td>diploid / 4.60%</td>
</tr>
<tr>
<td>29</td>
<td>aneuploid / 1.28</td>
<td>diploid / 3.90%</td>
</tr>
<tr>
<td>35</td>
<td>aneuploid / 1.33 and 2.22‡</td>
<td>diploid / 7.30%</td>
</tr>
<tr>
<td>39</td>
<td>aneuploid / 1.30</td>
<td>diploid / 8.00%</td>
</tr>
</tbody>
</table>

* DNA index, † Coefficient of variation, H=heterogeneity, ‡ Polyploid tumour.
Discrepancies between FCM and ICM

There were 13 discordant cases. As can be seen from Table 2, 12 of the aneuploid populations detected by ICM were not identified by FCM. One sample (case 35) clearly had polyploid peaks on the image histogram (Figure 1), whereas the flow histogram did not have peaks other than the G0/G1 peak. Another sample (case 25) showed a marked heterogeneity between different ICM measurements that was not present in the flow histogram. The DNA indices were 1.17 and 2.07 (G2/M 16.8%). This tumour was classified as aneuploid/tetraploid.

Of the 9 tumours aneuploid by FCM, ICM results corresponded in 8 of these cases. One tumour was recognised as aneuploid (DI=1.09) by FCM, no corresponding population was detected in the image histogram (CV=10.0).

![Figure 1. ICM histogram from a polyploid population. The first peak is defined as the diploid cell population and the two distinct additional peaks represent prominent aneuploid cell populations. The total number of analyzed nuclei was 847.](image)

Uninterpretable cases (n=14)

Although 10 cases were uninterpretable by ICM, the DNA content could be determined through FCM in all these cases and 4 additional aneuploid stemlines were recognised (Table 3). Interestingly, the DNA indices were all in the near-diploid region. Of the 4 uninterpretable cases by FCM, ICM detected one aneuploid population (DI=1.49).
Table 3. Aneuploid populations detected by flow cytometry (FCM), whereas image cytometry (ICM) histogram was uninterpretable.

<table>
<thead>
<tr>
<th>Case No</th>
<th>FCM / DI*</th>
<th>ICM / CV†</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>aneuploid / 1.13</td>
<td>uninterpretable / 10.10%‡</td>
</tr>
<tr>
<td>11</td>
<td>aneuploid / 1.23</td>
<td>uninterpretable / 14.20%‡</td>
</tr>
<tr>
<td>16</td>
<td>aneuploid / 1.10</td>
<td>uninterpretable §</td>
</tr>
<tr>
<td>44</td>
<td>aneuploid / 1.19</td>
<td>uninterpretable / 11.00%‡</td>
</tr>
</tbody>
</table>

* DNA index, † Coefficient of variation, ‡ Histograms with a coefficient of variation more than 10% were regarded as uninterpretable, § Image not possible due to the poor quality of the material

Table 4. Characteristics and overall incidence of aneuploidy of the 44 patients with DBDC, detected by flow cytometry or image cytometry.

<table>
<thead>
<tr>
<th>Tumour size*</th>
<th>No. of samples (n=44)</th>
<th>Aneuploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 2 cm</td>
<td>18</td>
<td>11 (61%)</td>
</tr>
<tr>
<td>&gt; 2 cm</td>
<td>18</td>
<td>11 (61%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microscopic resection margins</th>
<th>No-involvement</th>
<th>Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>8</td>
<td>4 (50%)</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>29</td>
<td>17 (58%)</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>7</td>
<td>5 (71%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lymph-nodes†</th>
<th>No-involvement</th>
<th>Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vasoinvasive growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>26</td>
<td>14 (54%)</td>
</tr>
<tr>
<td>Present</td>
<td>18</td>
<td>12 (67%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Perineural invasion‡</th>
<th>Absent</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>18</td>
</tr>
</tbody>
</table>

* From 36 patients the tumour size could be obtained, † The mean number of lymph node samples per tumour was 10, ‡ From one patient this information was missing

Prognosis
Among the 44 patients with DBDC in this study, 3 patients died after surgery (hospital mortality 7%) due to septic complications and were not included in the survival study. One patient was lost to follow-up after 2 years and was treated as a censored event at that time, leaving 40 cases in which follow-up information was complete. The medical records
or autopsies revealed that the cause of death in all patients who died during the follow-up period was recurrent tumour. In addition to surgical resection two patients received external beam radiation therapy and one patient received both chemotherapy and radiation therapy. No correlation of DNA-ploidy by FCM, or ICM, or a combination of FCM and ICM with survival time could be found, log rank test p=0.80, p=0.35, and p=0.54, respectively. In Table 4 the relationship of DNA-ploidy with several clinical-pathological criteria is shown. It is of interest that a trend towards a more aggressive phenotype of the aneuploid populations can be noted.

DISCUSSION

The incidence of aneuploidy in 44 DBDCs, combining both techniques of flow and image cytometry, was 59%. Because of the low incidence of this kind of tumor, comparable studies are limited and were performed on only a small number of patients. Jorba et al.27 analysed 8 DBDCs by FCM and found 2 aneuploid populations and Sciallero et al.28 detected 3 aneuploid tumours in 5 DBDCs, also by FCM. Comparable ICM studies on DBDCs were not found in literature. The contribution of ICM pertained to cell populations that were not identified by flow cytometry is responsible for the high incidence of DNA aneuploid tumours detected in this study. Our result is comparable to the incidence of aneuploid stemlines found in the other periampullary carcinomas, i.e. pancreatic carcinomas629,30 and ampullary carcinomas28,31.

Our results showed a moderate agreement between the two methods: there was concordance in 17 of the 30 cases where results could be compared (14 samples yielded uninterpretable results). By using the strict CV criteria described above and despite repeated measurements, 9% of the flow and 23% of the image histograms were considered uninterpretable. Weger et al.32 also observed methodological ICM problems in 1/4 of the cases in formalin-fixed paraffin-embedded material from pancreatic adenocarcinoma. Since all slides for ICM in this study were prepared from the same cell suspension used for FCM, no sampling area differences occurred. Eventually, five aneuploid populations were identified by FCM but were either not recognised or uninterpretable by ICM. One case showed a result that probably represented a tumour in which the abnormal population was missed by ICM simply because of the inadequate sampling or the higher CV. The other 4 cases had an unacceptably high CV or persistent debris. Interestingly, the DI of the aneuploid stemline was in all these cases in the near-diploid region. Due to the smaller number of cells analysed, ICM histograms tend to have lower resolution and higher CV compared to FCM histograms33,34, which makes it more difficult to distinguish near-diploid aneuploid tumours from diploid populations33,34. This was a possible source of error in DNA measurement by ICM in this study.

In addition, twenty seven cases (67%) were diploid using FCM. ICM was used in all these cases and it is of interest, therefore, that the diploid mode was confirmed in 9 cases, whereas 12 cases were reclassified as aneuploid (6 cases were not interpretable). FCM
DNA measurements requires a quantitative DNA abnormality of at least 5% to 10% of total cell population for detection. If only small numbers of malignant cells are present, ICM offers the ability to select and define the cell populations based on nuclear morphology. Like others, we preferentially selected intact nuclei, but did not attempt to distinguish benign from malignant cells based on the Feulgen-stained slides. Nuclei that were damaged by the process of fixation, embedding, dewaxing and trypsin digestion in paraffin-embedded tissue, were rejected as were overlapping nuclei. The resulting image histogram is more representative of the tumour itself and ICM, therefore, more sensitive than FCM in detecting small aneuploid populations. Although the admixture of non-tumour cells was reduced using microdissection of the histologically relevant tissue, dilution of an aneuploid tumour cell population by inflammatory or other non neoplastic cells, which is common in DBDCs, may have resulted in a diploid pattern by flow cytometry. Because dilution is not a factor in ICM, the contribution of ICM pertained to cell populations that were not identified by FCM. As a result, the detection rate of aneuploid tumour populations in positive cases was greater by ICM than by FCM, and we agree with others that ICM is more sensitive in detecting aneuploid populations. In addition, ICM provides a permanent record and also requires less sample tissue than FCM, which could be a useful adjunct to brush cytology of distal bile duct tumours.

The prognosis for DBDC is dismal and radical resection, when possible, is the only curative option. The high incidence of positive tumour margins in this group of patients, i.e. 43%, reflects the invasive characteristics of DBDC. It is often difficult to differentiate DBDC from the other periampullary carcinomas preoperatively or by histological classification. Disagreements can arise among pathologists reviewing the same slides, both in grading and typing of a specific tumour, leading to different estimations of the patient's prognosis. It would seem desirable therefore to use objective criteria to provide prognostic information. A fundamental difference in biological behaviour in various tumours with either a diploid or aneuploid DNA content has been found in previous quantitative DNA studies. In pancreatic carcinomas, gallbladder carcinomas, and ampullary carcinomas, the published results conflict about the prognostic value of ploidy status. Possible explanations of the many conflicting reports between laboratories concerning the prognostic value of DNA-ploidy include (1) differences that appear when a different number of nuclei are sampled; (2) intra-tumour heterogeneity, and the need for multiple sampling of solid tumours to obtain accurate DNA content measurements; (3) the number of cases studied; (4) the limitations of paraffin-embedded material by increased debris and wide CVs; (5) the value of the G2/M fraction used to classify a given tumour as tetraploid; (6) difficulties in the interpretation of image and flow histograms; (7) differences in the type of treatment given to the patients, which might have conferred any individual difference in survival. In this study, we found no correlation between ploidy by image, flow, or a combination of both and survival in a limited number of DBDCs, confirming the result of our previous study.
Although prognostic implications were not apparent, the degree of tumour aneuploidy was related to the histopathologic parameters.

In conclusion, a definite percentage of DBDCs (59%) are aneuploid. Certain aneuploid populations, however, may be missed when only one of the two techniques, i.e. ICM or FCM, is used. In agreement with others\textsuperscript{14,25,50}, we find that most of the discordant cases are due to the dilution of aneuploid cell populations by diploid cells present in the FCM cell suspension and that the combination of both techniques is superior to each of them individually. In DBDC, however, the significance of aneuploidy appears to be limited. No difference in prognosis between diploid and aneuploid tumours could be found, and we, therefore, believe that analysis of DNA-ploidy in patients with DBDC does not improve the ability to assess the prognosis of the patient.

REFERENCES


