The diagnostic and prognostic value of genetic aberrations in resectable distal bile duct cancer
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Chapter 3

Prognostic value of cell proliferation (Ki-67 antigen) and nuclear DNA content in clinically resectable distal bile duct carcinoma

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ABSTRACT

**Background.** The aim of this study was to investigate the prognostic value of cell proliferation (Ki-67 antigen) and DNA content in patients resected for distal bile duct carcinoma (DBDC).

**Methods.** Formalin-fixed tumor specimens of 35 patients with resected DBDC and a long-term clinical follow-up were analyzed. MIB-1 antibody was used for Ki-67 antigen detection to determine the proportion of proliferating cells. DNA content was measured using flow cytometry.

**Results.** A significant correlation was found between a low MIB-1 index (<20%) and survival (p<0.05). Of the 35 tumor specimens, 34 specimens were evaluable by flow cytometry: 22 carcinomas were diploid (65%), and 12 were aneuploid (35%). The median DNA index of aneuploid tumors was 1.36 (range 1.09 to 1.76). No correlation of DNA-ploidy with survival time was found.

**Conclusion.** In contrast to DNA-ploidy pattern, Ki-67 antigen expression showed prognostic significance in resectable DBDC. A Ki-67 positive ratio of ≤20% was associated with decreased survival time.

INTRODUCTION

Malignant neoplasms arising from the distal bile duct are uncommon tumors that are located between the superior border of the pancreas and the ampulla. Because of their intrapancreatic location they are defined as periampullary carcinomas, a group that also includes carcinomas arising from the pancreatic duct. Within the group of extrahepatic bile duct carcinomas, distal bile duct carcinomas (DBDC) commonly have a prognosis different from that of proximal bile duct carcinomas: the average 5-year survival rates of resected, proximal bile duct carcinomas range from 11% to 19% whereas for distal bile duct carcinomas, survival is reported to be between 24% and 33%. This striking difference in prognosis suggests not only an anatomic distinction between distal and proximal bile duct carcinomas, but also a different biologic predisposition.

Proliferation rate is a feature of tumors that has been used to determine their malignant potential. Gerdes et al. described the preparation of a mouse monoclonal antibody, Ki-67, that recognized a human intracellular antigen that is present in proliferating cells, but absent in resting cells. This antigen is expressed during the whole cell cycle, with the exception of the G_0 phase or early G_1 phase, and has proved useful in the evaluation of the proliferative activity of tumors. Several studies have focussed on the relationship between the growth fraction of different tumors and various other prognostic variables, as reviewed by Brown et al. Because the application of Ki-67 antibody is restricted to fresh material, a new antibody, designated MIB-1, was raised, enabling the detection of Ki-67 antigen in formalin-fixed, paraffin-embedded tissue.
Most human malignant cells carry detectable chromosomal anomalies, and the presence of these abnormalities is strong evidence for neoplasia. Assessment of nuclear DNA content in fresh or archival material allows detection of abnormal chromosomes and characterization of malignant tumors. DNA-ploidy has been shown to be an important prognostic factor in pancreatic carcinoma and in resectable cancer of the ampulla of Vater. In a previous study, we have reported a positive relationship between survival rate and DNA-ploidy in carcinoma of the proximal bile duct (Klatskin tumors). A significant difference was found between diploid and aneuploid tumors, with the aneuploid tumor correlating with a less favorable prognosis. Studies on DNA-ploidy and survival in gallbladder carcinoma have obtained the opposite results, however.

The aim of this study was to assess the degree of cell proliferation and DNA aneuploidy as prognostic factors in resectable DBDC. To this end, the expression of the cell cycle associated antigen Ki-67 and the DNA-ploidy pattern were determined in 35 patients who had undergone pancreatoduodenectomy (Whipple resection) for a DBDC. The results of Ki-67 antigen expression and DNA content were investigated in conjunction with survival time.

MATERIALS AND METHODS

Preparation of Tissue
Histologic paraffin-embedded tumor material from 35 patients with DBDC was studied. Patients from 1985 until 1992 were included so that a 5-year follow-up could be obtained. All patients were treated by subtotal pancreatoduodenectomy (Whipple procedure) at the Academic Medical Center, Amsterdam, The Netherlands. Of these 35 patients, 28 were male and 7 female, with a mean age of 59 years (range, 37 to 73 years), and clinical and histopathologic reevaluation confirmed that all tumors were DBDCs, located in the head of the pancreas. In large tumors, the main bulk of the tumor determined the site of origin. Patients with pancreatic or ampullary adenocarcinomas were excluded from this study.

To confirm the presence of bile duct cancer cells, 4 μm sections were cut for staining with hematoxylin and eosin. The pathologist selected the densest area of cancer cells in at least three tissue blocks per tumor. From these tissue blocks one to three 50 μm thick sections were cut for DNA measurements; additional 4 μm sections were used for immunohistochemical staining with the MIB-1 antibody. Ten extra samples of non-tumor areas of the resected specimens of the patients were stained for Ki-67 as controls.

Histopathologic examination
The following histopathologic features were assessed for each patient: surgical resection margins; pT-category; degree of tumor differentiation; tumor size; lymph node involvement; perineural invasion; and vasoinvasive growth. The mean number of lymph node samples per tumor was 10.
Immunohistochemical staining

Immunohistochemical studies were performed on paraffin-embedded tissue sections using monoclonal antibody MIB1, by means of the avidin-biotin-peroxydase complex technique. Paraffin sections (4 μm thick) were put on coated slides and were dewaxed and rehydrated in series of graded alcohol. After microwave irradiation, according to the modified method of Cattoretti et al., the slides were washed twice with phosphate-buffered saline (PBS) and stained according to the ABC method.

After a 1-hour incubation at room temperature with primary mouse monoclonal antibody MIB-1 (dilution 1:100, Immunotech Marseilles, France, 0505), biotinated rabbit anti-mouse IgG (Dako, E0413; diluted 1:200) was used as a secondary antibody. The immunoreaction was visualized by using the avidin-biotin complex (Strept ABC complex HRP, Dako A/S, Denmark, K377) method. Following 3,3'-diaminobenzidine, we used tetrahydrochloride in 0.3% hydrogen peroxide as a chromogen. The sections were counterstained with hematoxylin and each section also had a negative control by using PBS instead of MIB-1. Each of the processed slides was investigated without previous clinical information by two independent observers, and the number of positive stained nuclei out of at least 1000 tumor cells was counted. The results were expressed as the MIB-1 index, which represents the ratio of the MIB-1 positive tumor cell nuclei divided by the total number of nuclei counted.

Cell preparation and DNA staining technique

A modification of the basic method of cell preparation as described by Hedley and coworkers was employed. The 50 μm sections were trimmed to exclude non-tumor (stromal) tissue as much as possible according to the area pointed out by the pathologist. 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 pH 7.6. After washing in PBS, nuclear DNA was stained with propidium iodide by the detergent/trypsin method described by Vindeløv et al. Before analysis, all samples were filtered through a 40-μm nylon mesh to remove any residual tissue fragments. The specimens were analyzed on a Becton Dickinson FACScan® (Mountainview, CA) equipped with a 15-mW, 488-nm argon ion laser, a minimum of 1 hour after staining. Data were accumulated in 256-channel resolution.

DNA measurement

Three to six paraffin-embedded tumor blocks were analyzed per tumor specimen. Data from at least 20,000 nuclei per sample were collected and analyzed with the use of computer software (CellFIT; Becton Dickinson Immunocytometry System®, CA, USA). According to the consensual guidelines, samples with coefficient of variation (CV) values higher than 8% were rejected from this study. Mean CV of the G0/G1 peak in diploid histograms was 5.2% (range 2.7 to 7.6%) and 4.8% (range 2.6 to 7.5%) of the G0/G1 aneuploid peak.
Samples were classified as diploid or aneuploid according to the DNA histogram. In spite of trimming, most samples contained inflammatory and stromal cell nuclei, providing an optimum internal diploid standard for the determination of the ploidy pattern\textsuperscript{20,21}. The channel number of the first peak in the histogram was defined as diploid $G_0/G_1$. Aneuploidy was defined as one or more distinct separate peaks compared with the $G_0/G_1$ and $G_2/M$ peaks of the diploid cell population. Shoulders on the diploid peak or suspicious aneuploid subpopulations were not included as aneuploid peaks. The DNA index (DI) was defined as the ratio of the mode (or mean) of the relative DNA content of the $G_0/G_1$ cells of the sample divided by the mode (or mean) of the relative DNA measurement of the diploid $G_0/G_1$ reference cells. No histograms suggesting the presence of a tetraploid stemline (>15% $G_2/M$) were encountered. Histogram classification was performed without previous knowledge of the pathologic or clinical data.

**Statistical analysis**

Survival curves were generated using the Kaplan-Meier method, and univariate survival comparisons were made using the log-rank test (SPSS statistical software). $p<0.05$ was regarded as statistically significant.

**RESULTS**

Histopathologic examination showed radical resections in 19 patients and nonradical resections in 16 patients. Two patients died post-operatively (6%) while in the hospital and were not included in the survival study. Follow-up information was complete in 97% of patients; one patient was lost to follow-up after 2 years. In addition to surgical resection, two patients received external beam radiation therapy and one patient received both chemotherapy and radiation therapy. The medical records or autopsies revealed that all of the patients who died during the follow-up period had died of recurrent tumor.

Tissue sections of all patients were available for immunostaining. The mean MIB-1 index of all tumor areas ranged from 1% to 44% (median, 14.8%) whereas in the 10 benign areas, the MIB-1 index did not exceed 5%. The typical staining pattern of one sample is shown in Figure 1. In 12 patients (34%), the MIB-1 index was ≥20%, and a significant correlation was observed between the MIB-1 index and survival ($p=0.035$) (Figure 2). The median survival of patients with a MIB-1 index lower than 20% was 23 months, as compared to 10 months for patients with a MIB-1 index of 20% or more. The risk ratio was 2.86, indicating that the high score group had a mortality risk nearly three times higher than that of the low score group (log rank test, $p=0.005$).
Figure 1. Immunoreactivity with the MIB-1 antibody in distal bile duct carcinoma, showing strong nuclear staining.

Figure 2 Cumulative survival (Kaplan Meier curves) of patients whose tumors had a MIB-1 index ≥20% compared with those that had a MIB-1 index <20%. Two patients, who died postoperatively while in the hospital, were not included in the survival study.
Only one specimen was considered not evaluable by flow cytometry, because the CV of the diploid peak repeatedly exceeded 8% in 4 different samples. Of the remaining 34 patients, 22 (65%) carcinomas were diploid by FCM and 12 (35%) were aneuploid (Table 1; Figure 3). The median DI of aneuploid tumors was 1.36 (range, 1.09 to 1.76). In 8 tumors the DNA histogram of the samples tested showed aneuploid histograms next to diploid histograms, revealing a DNA heterogeneity rate of 23% (8/34). No correlation of DNA-ploidy with survival time could be found (log rank test, p=0.62), nor could it be found after stratification for radicality of the resection. Median survival of patients with diploid and aneuploid tumors was 20 and 21 months, respectively.

Table 1. Clinical data of 35 patients who underwent a Whipple resection for distal bile duct carcinoma.

<table>
<thead>
<tr>
<th>Patients (n=35)*</th>
<th>Diploid (n=22)</th>
<th>Aneuploid (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (years)</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>male*</td>
<td>28 (80%)</td>
<td>18</td>
</tr>
<tr>
<td>female</td>
<td>7 (20%)</td>
<td>4</td>
</tr>
<tr>
<td>Operative mortality within 30 days*</td>
<td>2 (6%)</td>
<td>1</td>
</tr>
<tr>
<td>Differentiation grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>well and moderate</td>
<td>30 (86%)</td>
<td>22</td>
</tr>
<tr>
<td>poorly*</td>
<td>5 (14%)</td>
<td>0</td>
</tr>
<tr>
<td>pT category*†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1</td>
<td>4 (11%)</td>
<td>3</td>
</tr>
<tr>
<td>pT2*</td>
<td>15 (43%)</td>
<td>8</td>
</tr>
<tr>
<td>pT3</td>
<td>16 (46%)</td>
<td>11</td>
</tr>
<tr>
<td>No. of patients with radical resection</td>
<td>19 (54%)</td>
<td>12</td>
</tr>
<tr>
<td>No. of patients with pos. lymph nodes*</td>
<td>16 (46%)</td>
<td>9</td>
</tr>
<tr>
<td>No. of patients with perineural invasion*</td>
<td>20 (57%)</td>
<td>13</td>
</tr>
<tr>
<td>No. of patients with vasoinvasive growth*</td>
<td>11 (31%)</td>
<td>6</td>
</tr>
<tr>
<td>Survival‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 year</td>
<td>23 (70%)</td>
<td></td>
</tr>
<tr>
<td>2 year</td>
<td>9 (27%)</td>
<td></td>
</tr>
<tr>
<td>5 year</td>
<td>7 (21%)</td>
<td></td>
</tr>
</tbody>
</table>

* Cases with one specimen not evaluable by flow cytometry
† According to UICC-TNM-classification of malignant tumours, fourth edition, 1997
‡ Two patients were not included because they died post-operatively

Radical resections (54%) were associated with statistically significant better survival (p<0.01). There was no significant difference in survival when sex (p=0.38), pT category
(p=0.30), tumor differentiation grade (p=0.10), vasoinvasive growth (p=0.13), perineural invasion (p=0.05), or lymph-node status (p=0.08) was considered separately (Table 1). Seven patients (22%) survived more than 5 years after microscopically radical resection of the DBDC. Of these long-term survivors, all but one had a MIB-1 index of less than 20% and 4 had diploid tumors (Table 2).

Table 2. Characteristics of the long survivors (> 5 year) in this study

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>No. of patients with radical resections</th>
<th>Ploidy</th>
<th>Ki67</th>
<th>* This patient had a MIB-1 index of 24%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Diploid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aneuploid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;20%</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥20%</td>
<td></td>
<td>1*</td>
</tr>
</tbody>
</table>

* Figure 3. FCM histogram from a 67-year-old man with poorly differentiated distal bile duct carcinoma. The first peak is defined as the diploid cell population (G0/G1 at 55), and the distinct additional peak (G2/G0 at 80) represents a prominent aneuploid cell population (DNA index 1.33). The total number of analyzed nuclei was 20,000.
DISCUSSION

Distinguishing histopathologically mitotic nuclei from nonproliferating nuclei requires experience and skill. Previous studies of periampullary carcinomas used flow cytometry to measure the growth rate of these tumors. This technique is difficult and time-consuming, however, and paraffin-embedded tissue is especially unsuitable because of the considerable amount of debris and the higher CVs often obtained. For this reason, it would be more appropriate to use a simpler but still reproducible method. Because Ki-67 antigen expression occurs in the whole cell cycle, with the exclusion of the G0 and early G1 phase, providing a direct measure of the growth fraction of the tissue, Ki-67 antigen is an accurate indicator of cell proliferation in histologic samples. The application of Ki-67 antibody previous was restricted to fresh frozen material, but since the production by Cattioretti et al. of a new, specific monoclonal antibody (MIB-1), it has become possible to detect the same antigen in paraffin-embedded tissue after microwave irradiation of the sections. This is of particular importance in DBDC, because these are rare tumors and require a long period for collection.

In the present study, a statistically significant correlation was found between decreased survival time and the extent of nuclear reactivity with Ki-67 antigen, using a cut-off point of 20%. This cut-off point was chosen because it selected for the highest quadrile of Ki-67 values. In a recent study of tumors of the gallbladder, ampulla, and common bile duct, significantly higher MIB-1 indices were similarly shown in neoplastic lesions, and were associated with a poor prognosis, although the authors did not define a cut-off point. Yamada et al. analyzed 21 patients who underwent resection of carcinoma of the middle and distal bile duct, but, in contrast to our study, they used a cut-off point of 10% to designate patients with a significantly better prognosis. Hall et al. also used a cut-off value of 20% Ki-67 positive cells in non-Hodgkin lymphoma and demonstrated that patients with a Ki-67 index of less than 20% survived significantly longer than did those with an index of more than 20%. Bouzubar et al. analyzed survival data of 124 patients with breast cancer who had been followed-up after mastectomy and found that Ki-67 nuclear staining of more than 20% was associated with a higher rate of recurrence of carcinoma. Thus, reported values of numerical cut-off points for survival may vary considerably.

Compared to other studies using fresh tissue, the maximum MIB-1 index found in this study was low. Because many antigenic epitopes do not survive the process of fixation, embedding, dewaxing, and trypsin digestion in paraffin-embedded tissue, the MIB-1 index was accordingly lower.

Flow cytometry (FCM) of the 34 carcinomas available for analysis revealed a 35% aneuploidy rate. Our results demonstrate that DNA-ploidy is not an important prognostic determinant in this type of malignancy. Due to the rarity of this tumor, comparable studies are limited. Yeaton et al., who used a cell-image processor, found 37% aneuploidy in 12 patients with DBDC. In a prospective study, Scialiero et al. found 2 aneuploid tumors among 5 patients, precluding any valid correlation. More recently,
Jorba et al.\textsuperscript{31} found 25% aneuploidy in 8 patients with DBDC, which correlated with poor median survival. However, in that study, only one sample of each tumor was examined. Caution must be used in interpreting the results of these studies in view of the rather small number of patients investigated. Although the DNA-ploidy estimations are in accordance with our results, it is possible that we have missed aneuploid stemlines for two reasons: (1) the sensitivity of FCM DNA measurements requires a quantitative DNA abnormality of at least 5\% to 10\% of total DNA content for detection\textsuperscript{32}, and (2) the CV of the diploid peak in the DNA histogram of paraffin-embedded tissue is greater than that of fresh tissue\textsuperscript{21}. As a result, the presence of near-diploid cytogenetic abnormalities in FCM distributions with wide CV $G_0/G_1$ peaks is not detected\textsuperscript{33}.

In a previous study\textsuperscript{14} in which 58 patients with carcinoma of the proximal bile duct were analysed, 52\% showed a DNA aneuploid pattern. Unlike the findings in the present study, aneuploidy in proximal bile duct carcinoma correlated with decreased survival. The reason for this fundamental discrepancy is not clear, but it may suggest that different genetic alterations determine the biologic behavior of the two tumors and supports the view that proximal and distal tumors form distinct entities. In this respect, the assessment of DNA-ploidy may be of value in the prognostic evaluation of proximal bile duct carcinoma but not of DBDC.

A wide range of aneuploidy has been reported for adenocarcinomas, especially in pancreatic cancer\textsuperscript{10,12,34,35}. Differences regarding the number of samples examined per tumor and the interpretation of DNA histograms make the data among laboratories difficult to compare. Intratumoral variation in DNA content has been found in several types of malignancies\textsuperscript{36,37}. In addition, there is a problem of heterogeneity of cells in carcinomas of the biliary tract as Suto et al.\textsuperscript{38} have pointed out. Therefore, we determined DNA-ploidy in at least 3 samples per tissue specimen, revealing a DNA-ploidy heterogeneity in 8 of 34 tumors (23\%).

The results clearly showed that, in addition to Ki-67 staining, radicality of resection also reached statistical significance as a determinant of long-term survival. A microscopically radical resection was achieved in only 54\% of patients, reflecting the biologic aggressiveness of DBDC. Tumor negative microscopic margins increased 5-year survival from 0\% to 39\%, and median survival from 13 months to 23 months.

In conclusion, assessment of mean Ki-67 antigen expression by MIB-1 staining of DBDC, proved an important indicator of clinical behavior in this type of cancer. A high proliferation score ($\geq$20\%) was associated with poor survival. DNA content, however, although it has gained importance as an independent prognostic variable in a number of tumors, had no prognostic value in resectable DBDC.
REFERENCES


