Genes and surgery in pancreatic cancer
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Micrometastases in bone marrow of patients with suspected pancreatic or ampullary cancer
ABSTRACT

Background
A lot of patients undergoing a radical resection for pancreatic cancer will ultimately suffer from distant metastases. Dissemination of malignant cells might have already taken place before surgery. Detection of these so-called micrometastases would improve pre-operative patient selection. This prospective study aimed to evaluate the detection of micrometastases in bone marrow of patients with suspected pancreatic or ampullary cancer and to determine their predictive value on overall survival.

Methods
Between December 1997 and December 1998, 35 patients (19 men, 42-77 years) with suspected pancreatic or ampullary cancer underwent diagnostic laparoscopy as a final staging procedure before exploration. Bone marrow was aspirated from the iliac crest at the beginning of laparoscopy. Mononuclear cells were isolated and stained using the specific monoclonal antibody CAM 5.2.

Results
Cytokeratin-positive cells were detected in 12/35 (34%) of all patients. In the 31 patients with a final diagnosis of carcinoma, a positive staining was found in 10/31 (32%) of the bone marrow aspirates. After a median follow-up of 17 months (2-24), 15/31 (48%) patients had died: 7/10 (70%) with and 8/21 (38%) without micrometastases (p<0.04). All four patients who turned out to have chronic pancreatitis, were alive without malignancy. In two of these four patients distinctly cytokeratin-positive cells were seen.

Conclusions
Micrometastases in bone marrow of patients with the final diagnosis pancreatic or ampullary carcinoma seem to predict a significantly shorter survival. However, clinical use of cytokeratin markers cannot be recommended at present, because false positive staining was found.
INTRODUCTION

The prognosis of patients with a pancreatic carcinoma is poor: the overall 5-year survival rate is 2%. Surgery is the only possible curative treatment modality. Still 70-80% of patients who undergo a radical resection by a pylorus preserving pancreatoduodenectomy (PPPD), will suffer from an incurable locoregional relapse or distant metastases. Locoregional relapse is mostly caused by an irradiical resection, which potentially can be shown by the presence of tumor in the dissection margins of the specimen. Distant metastases ultimately depend on dissemination of malignant cells, which has possibly already taken place before primary surgery. These so-called micrometastases are not detectable with conventional diagnostic tools. The ability to detect micrometastases would improve pre-operative patient selection and could guide future multimodality treatment. With the development of sensitive immunocytochemical methods, micrometastatic cells can now be directly assessed. The analysis makes use of monoclonal antibodies against cytokeratins, components of the cytoskeleton of all epithelial cells. Against the exclusively mesenchymal background of the bone marrow, micrometastases of epithelial tumors can be detected with great precision and sensitivity. In patients with breast, colon and lung carcinomas, the presence of these cells influenced the prognosis.

Few studies concern the presence of epithelial cells in bone marrow of patients with pancreatic and ampullary cancer, a tumor rarely metastasizing to the skeleton. The variation in detection rate of epithelial cells which is found in these studies (19%-61%), has raised doubts about the significance of the presence of such disseminated cells in pancreatic cancer. The aim of this study was to evaluate the possibility to detect micrometastatic tumor cells in bone marrow of patients suspected of having a pancreatic or ampullary carcinoma, and to determine their predictive value on overall survival.

METHODS

Patients

Between December 1997 and December 1998, 35 patients who were admitted to the Academic Medical Center in Amsterdam with a suspected tumor in the pancreatic head region participated in this study after informed consent was obtained. A patient was included when extensive radiological staging consisting of computed tomography scans, ultrasonography combined with Doppler, and endoscopic retrograde cholangiopancreatography had shown that the tumor probably was resectable. A patient was excluded when preoperatively, before the laparoscopy, histologically proven distant metastases were found. All patients underwent diagnostic laparoscopy as a final staging procedure several weeks prior to a planned explorative laparotomy. In two patients metastases were proven during laparoscopy and the remaining 33 patients underwent exploration for a pancreatic head tumor considered possibly resectable after laparoscopy. Because
of the heterogeneity in the final diagnosis and operative treatment in this patient group, a division in four subgroups was made (table 1):

1. Patients (n=14) who underwent a pylorus preserving pancreatoduodenectomy (PPPD) for a histologically proven adenocarcinoma of the pancreatic head
2. Patients (n=13) who underwent a palliative drainage procedure by surgical bypass (n=11) or stent (n=2). Of the 11 patients who underwent a surgical bypass, 7 patients were diagnosed with a pancreatic head carcinoma, and 4 patients with an ampullary carcinoma. One of the 2 patients who underwent a stenting procedure was diagnosed with an ampullary carcinoma, the other with a carcinoma of the body of the pancreas. In 9 of the 13 patients, irresectability of the tumour was due to local ingrowth, and in 4 patients the tumour could not be resected due to the finding of distant metastases during laparoscopy (n=2) or laparotomy (n=2). In case of a suspicion of local ingrowth during laparoscopy, an explorative laparotomy was still performed in order to obtain histological proof for the ingrowth.
3. Patients (n=4) who underwent a PPPD for a histologically proven ampullary carcinoma. Because this disease is known to have a better prognosis than an adenocarcinoma of the pancreatic head, we distinguished this subgroup.
4. Patients (n=4) who underwent a PPPD for what appeared to be a benign disease after histopathological examination

Bone marrow samples

After the induction of general anesthesia and before the start of the laparoscopy, all patients were subjected to aspiration of 10 ml bone marrow. This was obtained from the upper iliac crest through an aspiration needle (Becton-Dickinson, New Jersey, USA). The aspirates were collected in heparin 100 IU/ml and mingled in a 1:1 volume with RPMI 1640 (Boehringer Ingelheim, Belgium). This was centrifuged through a Ficoll-Hypaque density gradient (Pharmacia, Freiburg, Germany, density 1.077 g/mol) at 600 g for 30 minutes. Mononuclear cells were deposited on glass slides (1.0x10⁶ cells per slide) by cytocentrifugation at 1200 rpm for 5 minutes. Two cytospins were stained according to the routine PAP/Giemsma method. Epithelial cells in the bone marrow were identified immunocytochemically by staining ten cytospins with the mouse monoclonal antibody CAM 5.2 (IgG₂κ, Becton-Dickinson) which is specific for intracellular cytokeratin components 8 and 18.

Analysis

The same pathologist examined all slides by bright-field microscopy. A CAM 5.2 slide was regarded as positive when specific cellular staining was seen. Because there was no background staining, there were no indeterminate results.
Table 1 Detection of cytokeratin (CK)-positive cells in the four subdivisions

<table>
<thead>
<tr>
<th></th>
<th>CK-positive bone marrow (n=12)</th>
<th>CK-negative bone marrow (n=23)</th>
<th>All patients (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPPD, cancer pancreas</td>
<td>4 (29%)</td>
<td>10 (71%)</td>
<td>14 (40%)</td>
</tr>
<tr>
<td>Palliation, cancer</td>
<td>4 (31%)</td>
<td>9 (69%)</td>
<td>13 (37%)</td>
</tr>
<tr>
<td>PPPD, cancer ampulla</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
<td>4 (11%)</td>
</tr>
<tr>
<td>PPPD, no cancer</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
<td>4 (11%)</td>
</tr>
</tbody>
</table>

Statistical analysis

For statistical analysis either the chi-square or, when appropriate, the Fisher’s exact test was used to compare categorical variables. We used the Mann-Whitney U test to assess the differences in the means. All tests were two-tailed and a p-value of less than 0.05 was considered as statistically significant. Life-table curves for the 31 patients with an adenocarcinoma of the pancreas or ampulla were constructed according to the Kaplan-Meier method. Survival distributions in the patients with bone marrow micrometastases and those without micrometastases were compared by use of the log-rank statistic. The number of patients appeared to be too small to perform a multivariate statistical analysis.

RESULTS

Bone marrow aspirates from 16 female and 19 male patients with a suspected tumor in the pancreatic head region and a median age of 63 (range 42-77) were examined immunocytochemically. Cytokeratin (CK)-positive cells were detected in the bone marrow aspirates of 12/35 (34%) of all included patients. Table 1 shows the distribution of the cytokeratin-positive bone marrow aspirates in the 4 subgroups. In the 31 patients with a final diagnosis of carcinoma, a positive staining was found in 10/31 (32%) of the bone marrow aspirates. In two of the four patients (50%) in whom the final, histological diagnosis after a resection was chronic pancreatitis, distinctive staining by the monoclonal antibody CAM 5.2 was seen. Figure 1 shows a positive cytospin of such patient without a malignancy.

Figure 1 Cytospin of a typically positive bone marrow sample stained with CAM 5.2 (magnification 10 x 160) (x7 page 257)
Table 2: Clinical characteristics of the patients with a suspected tumor in the pancreatic head region, according to the presence or absence of occult metastatic cells in bone marrow

<table>
<thead>
<tr>
<th></th>
<th>CK-positive bone marrow (n=12)</th>
<th>CK-negative bone marrow (n=23)</th>
<th>All Patients (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age - yr *</td>
<td>61 (42%)</td>
<td>63 (48%)</td>
<td>63 (48%)</td>
</tr>
<tr>
<td>Sex:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>5 (42%)</td>
<td>11 (48%)</td>
<td>16 (48%)</td>
</tr>
<tr>
<td>M</td>
<td>7 (58%)</td>
<td>12 (52%)</td>
<td>19 (52%)</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resection - R₀ †</td>
<td>7 (58%)</td>
<td>10 (44%)</td>
<td>17 (50%)</td>
</tr>
<tr>
<td>Resection - R₁₂ †</td>
<td>1 (8%)</td>
<td>4 (17%)</td>
<td>5 (14%)</td>
</tr>
<tr>
<td>Palliative drainage</td>
<td>4 (34%)</td>
<td>9 (39%)</td>
<td>13 (39%)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malignant</td>
<td>10 (83%)</td>
<td>21 (91%)</td>
<td>31 (91%)</td>
</tr>
<tr>
<td>Non-malignant</td>
<td>2 (17%)</td>
<td>2 (9%)</td>
<td>4 (9%)</td>
</tr>
<tr>
<td>Tumor characteristics (UICC'97)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₁ (&lt;2cm)</td>
<td>4 (40%)</td>
<td>7 (33%)</td>
<td>11 (33%)</td>
</tr>
<tr>
<td>T₂ (&gt;2cm)</td>
<td>1 (10%)</td>
<td>5 (24%)</td>
<td>6 (24%)</td>
</tr>
<tr>
<td>T₃</td>
<td>3 (30%)</td>
<td>7 (33%)</td>
<td>10 (33%)</td>
</tr>
<tr>
<td>T₄</td>
<td>2 (20%)</td>
<td>2 (10%)</td>
<td>4 (10%)</td>
</tr>
<tr>
<td>Lymph Node Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N₀</td>
<td>5 (50%)</td>
<td>12 (57%)</td>
<td>17 (57%)</td>
</tr>
<tr>
<td>N₁</td>
<td>5 (50%)</td>
<td>9 (43%)</td>
<td>14 (43%)</td>
</tr>
<tr>
<td>Distant Metastases (liver)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M₀</td>
<td>8 (80%)</td>
<td>21 (100%)</td>
<td>29 (100%)</td>
</tr>
<tr>
<td>M₁</td>
<td>2 (20%)</td>
<td>0 (0%)</td>
<td>2 (0%)</td>
</tr>
</tbody>
</table>

UICC: International Union Against Cancer. * Age range 42-77 years
† R₀: no residual disease; R₁₂: microscopic/macroscopic residual disease

The clinical characteristics of the study population divided in CK-positive and CK-negative bone marrow aspirates are shown in table 2. No differences were found between the two groups regarding either tumor size (p=0.71), nodal status (p=0.77) or resection rate, respectively 8/12 (67%) in the CK-positive and 14/23 (61%) in the CK-negative bone marrow samples (p=1.0). The evaluation of patients with radical operations (R₀ resection) showed that 7/17 (41%) of the patients already had micrometastatic tumor cells in the bone marrow at the time of operation. Liver metastases were found in 17% (2/12) of patients with CK-positive bone marrow, whereas no patients with CK-negative bone marrow appeared to have liver metastases.

After a median follow-up of 17 months (2-24), 15/31 (48%) patients with pancreatic carcinoma had died: 7/10 (70%) of the patients with, and 8/21 (38%) of the patients without micrometastases. The median survivals in the different groups are summarized in table 3. The Kaplan-Meier survival analysis of the 31 patients with carcinoma of either pancreas or ampulla showed that cytokeratin
positive patients had a reduced overall survival (p<0.04) (figure 2). Including the four patients with a carcinoma of the ampulla does not influence the significant reduced survival. A comparison in survival in those 18 patients who underwent a PPPD for carcinoma, reveals a significantly worse prognosis in the patients with than in the patients without cytokeratin positive cells (p=0.04). Regarding the three subgroups with pancreatic carcinoma separately, a trend is seen in the correlation between the presence of cytokeratin positive cells and the survival, although no significance is reached. The four patients with a histologically proven benign disease of the pancreas were still alive after a median follow up of 22 months without signs of a malignancy.
The present study provides evidence that the finding of occult micrometastases, which can be detected in bone marrow by immunocytochemical analysis, may be a prognostic factor for survival in patients with pancreatic and ampullary cancer. It also indicates that the results must be interpreted with caution since specific positive staining by the monoclonal antibody CAM 5.2 was seen in two patients who turned out to have a pancreatitis without malignancy. Although it has been suggested that surgery is clearly palliative in essentially all patients with a pancreatic or ampullary carcinoma, at this moment resection offers the only hope on long-term survival. The high incidence of metastatic spread at the time of diagnosis not only shows the need for additional therapy, but also the importance of recognition of disseminated cancer cells in an early stage of the disease. Immunocytoelectrical techniques have made this early detection possible in the bone marrow of various cancer patients. Bone marrow is an optimal site to look for micrometastases because of its large blood supply, rich cellular repository, and mesenchymal nature, in which CK-positive cells are easily identifiable. Prognostic relevance of these cells has been shown mostly in studies performed with breast cancer patients using one or various specific antibodies for epithelial cells to detect dissemination of tumour in the bone marrow at the time of operation. So far, only few studies investigated the prognostic value of epithelial cells in the bone marrow of peri-pancreatic cancer and their detection rate varies widely from 19%-61%. Although Juhl et al. found CK-positive bone marrow aspirations in 61% of pancreatic carcinoma patients, they did not find a significant correlation with the survival, probably partly due to the short follow up period of 12 months (p=0.17). In only two studies survival analyses revealed that the presence of epithelial-positive cells in bone marrow was associated with a reduced overall survival. Although in our study the number of patients is relatively small and the follow-up period (median 17 months) limited, a significantly worse prognosis is found for patients with CK-positive cells in their bone marrow, considering all 31 peri-pancreatic cancer patients as well as those (n=18) who underwent a PPPD for carcinoma. This possibility of detecting micrometastases, assuming CK-positive cells are, could improve patient selection for surgery in general and for resection in particular. The lack of a correlation between respectively tumour size, nodal status, resection rate and the incidence of CK-positive cells in our study can be explained by the small sample size, or by the suggestion by Pantel et al. that cells are kept in a dormant state for years, but still have the potential to develop distant metastases. Similar behaviour of tumour cells has been found for other epithelial tumours, such as non-small-cell lung cancers. False positive findings of micrometastases in bone marrow however have also been reported. An explanation could be that CK-positive cells are morphologically sometimes undistinguishable from hematopoietic bone marrow cells. Juhl et al. detected in 9/28 patients (32%) without a malignancy a positive staining by the cytokeratin marker C54-0. In a more recent study from
the same institution with an identical panel of six different antibodies and a higher number of patients, false positive findings in the control group were found in 4%. This illustrates a large variation in the detection of false positive samples.

The controversy over the prognostic relevance of the presence or absence of cytokeratin-positive cells in the marrow may also be explained by the use of different antibodies, staining techniques and criteria for defining positively stained cells. Considering this last issue, our criterion for positivity, i.e. specific cellular staining, does not influence the amount of false positive findings, or consequently the prognostic relevance. After all, in our study distinctly cellular staining was seen in a patient with pancreatitis (figure 1). Currently, a prospective, stratified trial is performed in our clinic to look for micrometastases in bone marrow of a control group, consisting of patients with a haematological disorder without evidence of malignancy. The detection rate of CK-positive bone marrow and consequently its prognostic relevance also seem to be influenced by the site of aspiration. This is described in a recent study on micrometastases in esophagogastric cancer, where the presence of stained cells in the marrow taken from rib segments was five times as high as from the iliac crest. This may relate to the quality of marrow obtained, to the foregut site of the primary tumor, but probably may also be attributed to other variables, such as the amount of marrow obtained and the different staining techniques that were used.

Beside the importance of the sensitivity of an immunocytochemical marker for detection, the specificity is at least as important concerning its clinical implication in the future. The advantage of early detection of micrometastases in bone marrow of patients having a pancreatic or ampullary tumour, could be better patient selection for a major operation like the PPPD, which still is the only possibly curative option, or for down-staging the tumour by preoperative chemotherapy. Therefore the consequence of false positive findings could be enormous - withholding the patient's chance of cure.

In conclusion, like previous studies, our data suggest that, by using an immunocytological approach, CK-positive cells become frequently detectable in the bone marrow of patients who are suspected of having a pancreatic or ampullary carcinoma. The occurrence of these micrometastases in patients with a final diagnosis of a malignancy correlates significantly with a shorter survival. As such, the detection of CK-positive cells defines a new risk category of pancreatic and ampullary cancer patients and may be useful in the decision whether systemic treatment is required and in monitoring its therapeutic efficacy. Another message that can be concluded from our study, is that clinical use of cytokeratin markers cannot be recommended in patients with suspected pancreatic cancer at present, because distinctive staining was seen in the bone marrow aspirate of patients with a pancreatitis. The results of this small and preliminary study need to be interpreted with caution and a more definite study would require a greater number of patients and longer follow-up.
REFERENCES


