Strong prognostic value of nodal and bone marrow micro-involvement in patients with pancreatic ductal carcinoma receiving no adjuvant chemotherapy

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Abstract

AIM: To study the prognostic value of adjuvant chemotherapy in patients with pancreatic, ductal adenocarcinoma.

METHODS: Lymph nodes from 106 patients with resectable pancreatic ductal adenocarcinoma were systematically sampled. A total of 318 lymph nodes classified histopathologically as tumor-free were examined using sensitive immunohistochemical assays. Forty-three (41%) of the 106 patients were staged as $pT_{1/2}$, 63 (59%) as $pT_{3/4}$, 51 (48%) as pN_0, and 55 (52%) as pN_1. The study population included 59 (56%) patients exhibiting $G_{1/2}$, and 47 (44%) patients with G_3 tumors. Patients received no adjuvant chemo- or radiation therapy and were followed up for a median of 12 (range: 3.5 to 139) mo.

RESULTS: Immunostaining with Ber-EP4 revealed nodal microinvolvement in lymph nodes classified as "tumor free" by conventional histopathology in 73 (69%) out of the 106 patients. Twenty-nine (57%) of 51 patients staged histopathologically as pN_0 had nodal microinvolvement. The five-year survival probability for pN0-patients was 54% for those without nodal microinvolvement and 0% for those with nodal microinvolvement. Cox-regression modeling revealed the independent prognostic effect of nodal microinvolvement on recurrence-free (relative risk 2.92, *P* = 0.005) and overall (relative risk 2.49, *P* = 0.009) survival.

CONCLUSION: The study reveals strong and independent prognostic significance of nodal microinvolvement in patients with pancreatic ductal adenocarcinoma who have received no adjuvant therapy. The addition of immunohistochemical findings to histopathology reports may help to improve risk stratification of patients with pancreatic cancer.

Yekebas EF, Bogoevski D, Bubenheim M, Link BC, Kaifi JT, Wachowiak R, Mann O, Kutup A, Cataldegirmen G, Wolfram L, Erbersdobler A, Klein C, Pantel K, Izbicki JR. Strong prognostic value of nodal and bone marrow micro-involvement in patients with pancreatic ductal carcinoma receiving no adjuvant chemotherapy. *World J Gastroenterol* 2006; 12(40): 6515-6521

http://www.wjgnet.com/1007-9327/12/6515.asp

INTRODUCTION

Pancreatic adenocarcinoma is the fifth leading cause of death among all malignancies[1], leading to approximately 40 000 deaths each year in Europe[2]. Reported probabilities of five-year survival after curative surgery are still below 10 percent[3]. Stage, grade and resection margin status are currently accepted as the most accurate pathologic variables predicting survival[4-10]. Pathologic staging only insufficiently reflects the individual risk to develop tumor recurrence which is even high in early tumor stages. Thus, effort continues to identify new prognosticators of tumor

relapse that indicate the need of adjuvant therapy.

Occult residual tumor disease is suggested when either bone marrow or lymph nodes from which tumor relapse may originate are affected by micrometastatic lesions undetectable by conventional histopathology[11]. The clinical significance of antibodies against tumor-associated targets both in lymph nodes[12-15] and in bone marrow[15,16] is still controversial[16-26]. Various monoclonal antibodies are in use for micrometastatic detection, thus contributing to the incongruity of data and validity of results. These assays have been rarely used in patients with pancreatic carcinoma[27-31]. Recently, our group showed that immunohistochemical staining with the monoclonal antibody Ber-EP4 is a sensitive and specific method for detecting isolated or clusters of tumor cells in lymph nodes from patients with lung[13], esophageal[25], or pancreatic carcinomas[28]. Ber-EP4 is an antibody against two glycopolypeptides of 34 and 49 kD on the surface and in the cytoplasm of all epithelial cells (except parietal cells, hepatocytes, and the superficial layers of squamous epithelium).

The present study was to increase our knowledge gained in the previous studies on lymph node micrometa stasis[25,28,31,32]. In non-small cell lung carcinoma[32], the risk to develop tumor relapse in pN1 patients is overall greater than in pN0 patients. However, we have shown that further risk stratification for patients with histopathological involvement may be performed according to their immunohistochemical status. Therefore, here we have extended our previously published study on lymph node-negative patients with pancreatic cancer[31] with patients staged as pN1 through conventional histology as well as the bone marrow data from those patients that gave us written consent. The primary aim of this study was to assess the role of immunohistochemically detectable micrometastases in lymph nodes of an unselected group of patients with "curatively" resected pancreatic ductal adenocarcinoma. The secondary aim was to assess whether lymph node microinvolvement is correlated to bone marrow micrometastasis and which of these two sites is a better indicator for tumor cell dissemination in pancreatic cancer.

MATERIALS AND METHODS

Patients and study design

The local ethical committee of Hamburg approved this study. Informed consent was obtained from all patients before inclusion in the study. Tumor samples, lymph nodes, and bone marrow aspirates of the upper iliac crest were collected from 487 patients with pancreatic and periampullary malignancies. Out of these patients, 171 (35%) had carcinomas of the papilla of Vater, 47 (10%) exhibited carcinoma of the distal common bile duct, and 269 (55%) had pancreatic carcinoma. Out of these 269 patients, 49 (18%) had neuroendocrine tumors and 220 (82%) had true pancreatic carcinoma.

Our study population included 106 patients with resectable pancreatic ductal adenocarcinoma who had undergone curative surgery and had given informed consent for immunohistochemical analysis of lymph

nodes. Patients with cystic malignancies (IPMN, cystadenocarcinoma), acinar cell and squamous cell carcinomas were not considered for this study. The most frequent surgical procedure was pancreatoduodenectomy. Lymph node dissection was performed as previously described by Pedrazzoli et al[33]. A total of 1643 lymph nodes were removed with a median number of 16 (range 7 to 38) lymph nodes per patient. Among all histopathologically negative lymph nodes, 318 were selected in a representative fashion as described most recently for subsequent immunohistochemical screening[31]. Tumor stage and grade were classified according to the 6th edition of the tumor-node-metastasis classification (TNM) of the International Union against Cancer[34] by investigators unaware of the immunohistochemical findings.

Follow-up evaluations at three-month intervals included a physical examination, abdominal ultrasonography, computed tomography of the abdomen and studies of tumor markers, i.e. carcinoembryonic antigen and CA 19-9. Out of all 106 patients studied, the vital status in 89 patients could be determined at the end of the study. Seventeen patients were excluded from the survival analysis because they were either censored or died within 90 d after surgery. From 3 patients, only information about the date of death but not of recurrence was available

Tissue preparation and immunohistochemical analysis

Lymph nodes were divided into two parts, one for conventional histopathology, the other was snap-frozen in liquid nitrogen within three hours after their removal and stored at -80℃ until use. Only histopathologically "tumor-free" lymph nodes were screened by immunohistochemistry with the anti-epithelial-cell monoclonal antibody Ber-EP4 (IgG1; Dako, Hamburg, Germany) as described previously[14]. Ber-EP4 is an antibody against two glycopolypeptides of 34 and 49 kD on the surface and in the cytoplasm of all epithelial cells (except parietal cells, hepatocytes, and the superficial layers of squamous epithelium). The antibody does not react with mesenchymal tissue, including lymphoid tissue[15,23].

Cryostat sections (5 to 6 μm thick) were cut at three different levels in each node and transferred onto glass slides treated with 3-triethoxysilylpropylamin (Merck, Darmstadt, Germany). One section of the sample obtained at each level was stained by the alkaline phosphatase-antialkaline phosphatase technique combined with the new fuchsine stain (Sena, Heidelberg, Germany) for the visualization reaction[23].

In 16 control patients with nonepithelial tumors or inflammatory diseases, lymph nodes were consistently stained negative. Sections of normal colon served as positive staining controls and isotype-matched, irrelevant murine monoclonal antibodies served as negative controls (purified immunoglobulin mouse myeloma protein for IgG1; Sigma, Deisenhofen, Germany).

The slides were evaluated in a blinded fashion by two observers working independently (D.B., J.T.K.). Minimal tumor cell involvement in a lymph node that was considered to be tumor-free by conventional histopathological staining was defined as the presence of
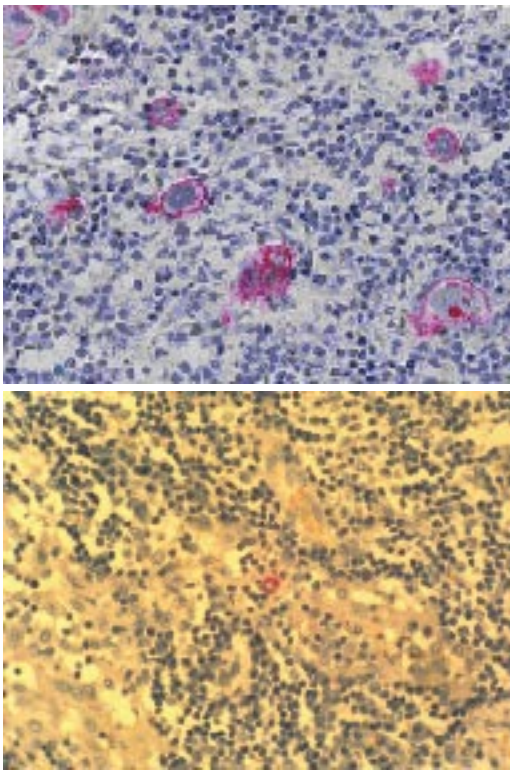
Figure 1 Immunohistochemically detectable nodal microinvolvement with monoclonal antibody Ber EP4 (×400).

Figure 2 Immunohistochemically detectable bone marrow microinvolvement with monoclonal antibody AE1/AE3 (×400).

one to ten positive cells in the body of the node (Figure 1). If more than 10 cells were detected (2 lymph nodes in two patients), a HE re-staining was conducted. Under routine histology both lymph nodes were judged as negative.

Aspirates of 4 to 8 mL of bone marrow from the iliac crest were obtained from those patients who gave additional written consent for sampling bone marrow (59 patients) and were processed as previously described[16]. The specimens were collected in heparin, and mononuclear cells isolated by density-gradient centrifugation through Ficoll-Hypaque (Pharmacia, Freiburg, Germany) at 400 r/min for 30 min, were deposited onto glass slides by cytocentrifugation at 150 r/min for 3 min. To detect tumor cells in bone marrow (Figure 2), we used the monoclonal antibody A45-B/B3 (IgG1; Micromet, Munich, Germany) that detects an epitope on a variety of cytokeratin components, including cytokeratins[14,15,18,20].

Statistical analysis

All statistical calculations concerning survival (overall and recurrence-free survival) were based on the group of 89 patients who were available for follow-up. The primary outcome measure was the five-year survival probability. Secondary outcomes were the incidence of local recurrence and distant metastases of the disease. Survival was calculated from the date of resection until the date of death from any cause. For patients lost to follow-up, data were censored on the date the patient was last seen alive. Associations between categorical variables were assessed using Fisher's exact test. Survival estimates were derived using the method proposed by Kaplan and Meier[35] and the log-rank test was used to assess differences in survival
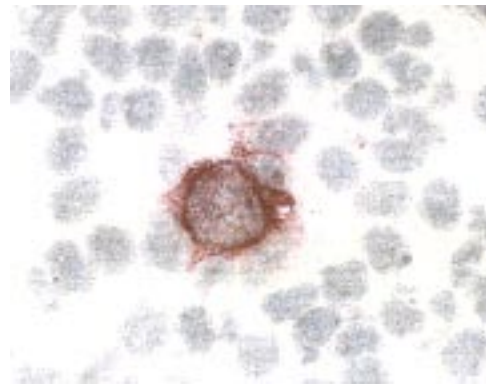
estimates among the groups. Point and interval estimates of the survival probabilities at 60 mo were calculated. For comparison purposes, log-rank test and exact stratified log-rank test were performed. Cox proportional-hazards modeling[36] was used to investigate and adjust the major prognostic and stratification factors. $P < 0.05$ was considered statistically significant.

Since this analysis was intended to be explorative, no adjustment for multiple testing was carried out.

RESULTS

Characteristics of patients and comparison of staining procedures

One hundred and six patients [47 (44%) women and 59 (56%) men] with pancreatic ductal adenocarcinoma were included in the study. Their mean age was 61 years (range 32 to 83 years, median 61 years). Table 1 shows the characteristics of patients and tumors.

A total of 318 lymph nodes classified to be "tumor-free" by conventional histopathology were analyzed. Positive cells in the sinuses, the lymphoid interstitium, or in both locations were found in 132 lymph nodes (42%). These 132 positive lymph nodes were found from 73 (69%) of the 106 patients by immunostaining. Whereas the presence of Ber-EP4 cells was significantly associated with nodal metastases (pN1) identified through conventional histopathology ($P = 0.012$), no correlation between tumor stage and tumor grade was found.

Survival

After an average observation period of 18 mo (range 3 to 137 mo, median 12 mo), the presence of nodal microinvolvement was associated with significantly reduced recurrence-free and overall survival probabilities. The Kaplan-Meyer overall survival curve for all patients who were stratified according to the presence or absence of occult tumor cells in lymph nodes showed a significant survival benefit for patients negative in immunohistochemistry (median not yet reached-NYR *vs* 13 mo; 2- year survival 66% *vs* 20%; 5-year survival 50% *vs* 0%) irrespective of the histopathological classification (pN0/pN1) of lymph nodes (log-rank test; $P < 0.0001$, Figure 3).

Table 1 Characteristics of patients and tumors		
Variable	*n*	Ber-EP4 positive cells in lymph nodes (*n*)
All patients	106	73
Male	59	42
Female	47	31
Primary Tumor		
Carcinoma in situ	1	0
pT1	6	3
pT2	36	26
pT3	59	40
pT4	4	4
Nodal status		
Negative (pN0)	51	29*
Positive (pN1)	55	44*
Tumor Grade		
Well differentiated (G1)	5	4
Moderately differentiated (G2)	54	35
Poorly differentiated (G3)	47	34
Resection margin		
Negative (R0)	90	59
Positive (R1)	16	14
Tumor cells in Bone marrow		
Yes	14	9
No	45	31
Not analysed	47	33

Nodal status was detected both histopathologically and immunohistochemically. Bone marrow micrometastases were detected by immunohistochemistry. $P = 0.035$.

The analysis of the subset of patients who were staged pN0 in conventional histopathology revealed significantly better survival rates in patients without occult tumor cells as compared with those with nodal microinvolvement (median NYR *vs* 17 mo; 2-year survival 70% *vs* 34%; 5-year survival 61% *vs* 0%; log-rank test; $P=0.012$, Figure 4). Patients without any nodal involvement, as excluded by both conventional histopathology and immunohistochemistry, had a five-year overall survival probability of 61% (standard error: 13%). In contrast, the five-year survival probability of pN0-patients with nodal microinvolvement resembled that of pN1-patients (log-rank test; $P = 0.059$, Figure 4) and in both groups no patient was still alive 5 years after surgery.

The predictive value of nodal microinvolvement was strengthened by the finding that pN1-patients who additionally had disseminated tumor cells in other lymph nodes classified as tumor-free by histopathology had shorter recurrence-free and overall survival probabilities than pN1-patients without occult tumor cells in immunohistochemistry (median survival 33 *vs* 10 mo; 2-year survival 69% *vs* 10%; 5-year survival 69% *vs* 0%; log rank test: $P = 0.004$ and $P = 0.049$, respectively, data not shown).

Although no statistical significance was reached, pN1-patients *without* nodal microinvolvement had better overall survival probabilities (median survival = 33 mo) than pN0-patients *with* nodal microinvolvement (median survival = 17 mo). This could in part confirm the hypothesis that
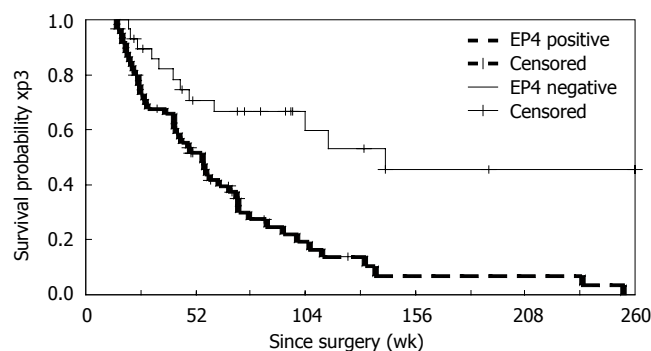

Figure 3 Overall survival according to the presence or absence of nodal microinvolvement in immunohistochemistry for patients alive at least 13 wk after surgery. Median: Not yet reached-NYR *vs* 13 mo; Mean: 81 (SD 15, 95% CI 52-109) *vs* 16 (SD 2, 95% CI 12-20); 2-yr overall survival 66% *vs* 20%; 5-yr overall survival 50% *vs* 0%.
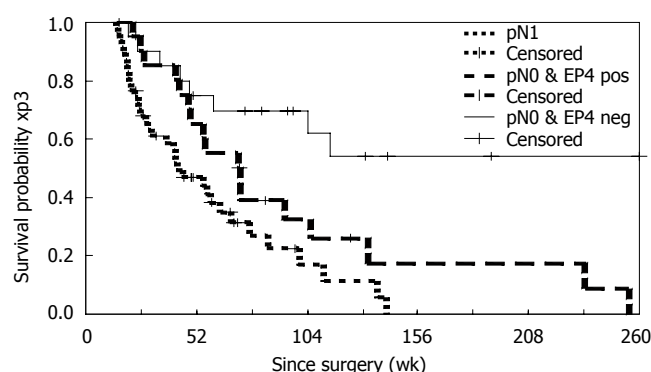

Figure 4 Overall survival according to the presence or absence of nodal metastases in conventional histopathology and immunohistochemistry for patients alive at least 13 wk after surgery. Median: Not yet reached-NYR *vs* 13 mo *vs* 10 mo; Mean: 90 (SD 15, 95% CI 60-119) *vs* 17 (SD 3, 95% CI 11-23) *vs* 14 (SD 2, 95% CI 7-13); 2-yr overall survival 66% *vs* 20%; 5-yr overall survival 50% *vs* 0%.

nodal microinvolvement in patients not burdened with nodal metastases detected through routine histology (pN0) might literally reflect systemic disease, whereas pN1-patients without nodal microinvolvement could be in fact treated as locally advanced disease. This should be discussed very cautiously since only 8 patients were included in the group of pN1-patients without nodal microinvolvement, but all of them were alive at the last follow-up.

Influence of resection margins
A total of 16 patients among the entire study population had positive resection margins (R1-status), only 2 of them belonged to the subset of pN0-patients without nodal microinvolvement. The remaining 14 patients with positive resection margins were either staged pN1 or had nodal microinvolvement which was as reported above, per se associated with a significantly worse prognosis. Due to this distribution of patients with R1-status, log-rank test might not have the sufficient power to assess the influence of the resection margins on overall survival. Therefore, no significant differences were found between patients with positive resection margins, as compared with those with negative resection margins ($P = 0.976$, data not shown).

Table 2 Cox regression analysis showing the presence of occult tumor cells in lymph nodes, tumor grade, and histopathologically detectable lymph node involvement as independent prognostic factors for disease-free and overall survival

Prognostic factor	Reference group	Relative risk	P	Lower bound	Upper bound
				95% confidence interval	
Recurrence-free (*n* = 86)					
Age at surgery		1.01	0.706	0.98	1.03
Sex	Male	1.42	0.223	0.81	2.49
Stage	pT1-pT2	0.87	0.652	0.48	1.57
Grade	G I - GII	3.14	0.000	1.74	5.68
Nodal involvement	pN0	1.66	0.112	0.89	3.10
Nodal microinvolvement (Ber-EP4)	Negative	2.92	0.005	1.39	6.13
Overall survival (*n* = 89)					
Age at surgery		1.00	0.996	0.97	1.03
Sex	Male	1.47	0.172	0.85	2.55
Stage	pT1-pT2	0.93	0.801	0.53	1.64
Grade	G I - GII	3.36	0.000	1.90	5.94
Nodal involvement	pN0	2.18	0.012	1.19	4.00
Nodal microinvolvement (Ber-EP4)	Negative	2.49	0.009	1.25	4.96

Influence of bone marrow micrometastases

Occult tumor cells were detected in 14 of 59 patients who were evaluated with respect to bone marrow micrometastases. No association was found between bone marrow and nodal microinvolvement (Fisher's exact test, $P = 0.75$). Among the 14 patients with bone marrow micrometastases, 9 (64%) had also nodal microinvolvement, whereas 5 (36%) did not. In turn, Ber-EP4-positive cells in lymph nodes were detected in 31 (69%) of the 45 patients with negative bone marrow findings (Table 1). Neither bone marrow micrometastases nor nodal microinvolvement was found in 14 patients. Here, we want to stress the finding (although again not significant due to the small number of patients) that those patients without any nodal involvement (negative both in histopathology and IHC) who had bone marrow micrometastases seemed to have considerably worse overall prognosis (mean survival time: 8 mo) than patients without (mean survival time: 75 mo). This also has to be discussed with considerable caution since we identified only 3 patients with bone marrow micrometastases (without any nodal involvement), although they all died within the first year unlike the other 8 patients without bone marrow micrometastases who were all alive on the last follow-up.

As regards the influence of bone marrow micrometastases alone, no significant differences were found with respect to relapse-free time ($P = 0.55$) and overall survival ($P = 0.14$), respectively. However, these results might be biased by the small number of patients analyzed.

Multivariate analysis

Apart from nodal involvement assessed either by histopathology or immunohistochemistry, the comparison of survival curves revealed also significant differences with respect to grading when G$_{1,2}$ tumors were compared to G$_3$ tumors (median survival time: 28 mo *vs* 12 mo; log-rank test: $P < 0001$).

Cox regression analysis identified the presence of occult tumor cells in lymph nodes, tumor grade, and histopathologically detectable lymph node involvement as

independent prognostic factors for disease-free and overall survival (Table 2). With respect to 5-year recurrence-free time, nodal microinvolvement had a relative risk of 2.92 (95% confidence interval: 1.39 to 6.13; $P = 0.005$), as compared with negative findings in immunohistochemistry. G3 tumors had a relative risk of 3.14 as compared with G1/2 tumors (95% confidence interval: 1.74 to 5.68; $P = 0.000$) with respect to recurrence-free time. A pN1-stage, as compared to pN0 patients, carried a relative risk of 2.18 (95% confidence interval: 1.19 to 4.0; $P = 0.012$) as to overall survival time, but had no significant influence on recurrence-free time (relative risk of 1.66; 95% confidence interval: 0.89 to 3.10; $P = 0.112$). Age, sex and tumor stage had no independent prognostic influence on recurrence-free or overall survival.

The analysis of the interaction between pN-status, nodal microinvolvement and grading did not reveal that the proportional assumption was violated. Hence, the Cox model appeared appropriate and grading followed by nodal microinvolvement remained the two most important prognostic variables also in the subset of pN0-patients.

DISCUSSION

The key finding of this investigation is that isolated tumor cells, detectable in lymph nodes by immunohistochemical analysis, are strong independent prognosticators in pancreatic ductal adenocarcinoma irrespective of the histopathological N-status. We analyzed patients who suffered from pancreatic ductal adenocarcinoma and did not receive any adjuvant chemoradiation or chemotherapy. Two subsets of patients could be identified and were classified as pN0 in conventional histopathology. One subset with a poor five-year survival probability of 0% which was close to that of patients with overt nodal involvement (pN$_1$), the other subset had a much better prognosis with a five-year survival probability of over 50% without nodal microinvolvement, suggesting that immunohistochemistry can confirm the cardinal importance of occult tumor cells for the separation of the

respective survival curves in pN0-patients.

Even in patients who were staged as pN1, the detection of occult tumor cells in "tumor-free" lymph nodes had prognostic significance. This finding is consistent with previous observations of our group showing that survival is significantly worsened in both esophageal[25] and non-small cell lung carcinoma[32] when histopathological pN1-status is accompanied with nodal microinvolvement. Basically, pN1-status in solid tumors is considered as a local disease which can be potentially cured with surgery, although it generally carries a higher risk of systemic dissemination than pN0-status. Therefore, the finding that pN1 patients with additional nodal microinvolvement in "tumor-free" lymph nodes apart from overt lymph node metastases had significantly shorter recurrence-free survival as compared with pN1-patients without occult tumor cells suggests that immunohistochemistry may help to identify different risk profiles in these patients.

The reliability of these immunohistochemical assays used so far for detection of nodal microinvolvement is questioned[16-26] and could be also hampered by a sampling error[25,37]. The sampling error might be influenced by the number of lymph nodes dissected during the course of pancreatoduodenectomy and assessed by immunohisto-chemistry, as well as the number of lymph node sections and the level of these sections within the lymph nodes. In our present study, the lymph node dissection was performed as previously described by Pedrazzoli et al[33]. A total of 1643 lymph nodes were removed with a median number of 16 (range 7 to 38) lymph nodes per patient. Thus, the first possible cause of a sampling error was diminished, considering the high median lymph node yield of 16 per patient. In another study (oesophagus carcinoma, not yet published) all lymph nodes dissected in the course of esophagectomy were immunohistochemically stained, unlike in this and some other previously published studies[25] where only 20% of all lymph nodes were stained, showing comparable results in terms of impact on overall and relapse-free survival. We also believe that 6 sections cut from 3 different levels of each lymph node are enough for proper access to the nodal microinvolvement. Analyzing more than 3 sections would not be routinely feasible, and the positive correlation between the result of our assay and clinical outcome indicates that examining 3 lymph node levels are sufficient. The results from this study are in the same line with those published earlier from our group[25], thus confirming that the random selection of lymph nodes for IHC is enough for access to the nodal microinvolvement.

The strong adverse influence of nodal microinvolvement on outcome was most likely the reason for the lack of prognostic significance of resection margins in this series. In the ESPAC-1 trial, the resection margin status ceased to be an independent, prognostic factor for overall survival when tumor grading and pN-status were co-variables in the regression modeling[38], suggesting that the unfavorable outcome linked with poorly differentiated tumors with nodal metastases can hardly be impaired by further adverse variables, e.g. R1-status. We therefore assume that the characteristics of patients with R1-status are considerably biased. This hypothesis is strengthened by our observation that out of the 16 patients with R1-status all except two had occult tumor cells in immunohisto-chemistry.

Our data also indicate primary dissemination of tumor cells into lymph nodes before blood-borne spread occurs, because only 22% of the patients with nodal microinvolvement had identifiable tumor cells in their bone marrow. The lack of a significant difference in recurrence-free time between patients with nodal microinvolvement alone and those with additional involvement of the bone marrow suggests that the key event in pancreatic cancer progression is the spread of tumor cells to the regional lymph nodes. Nodal microinvolvement seems to indicate a systemic disease in pancreatic carcinoma much more accurately than occult tumor cells in bone marrow. However, these results have to be interpreted cautiously because bone marrow findings may have been biased by the fact that only a subset of patients was analyzed. Therefore, the influence of bone marrow microinvolvement on the outcome of patients with pancreatic carcinoma needs to be clarified in future studies.

Although chemoradiation and/or chemotherapy for adjuvant treatment of pancreatic carcinoma may have severe side effects[39], in common clinical practice, it is in most instances applied irrespective of tumor stage. This reflects the distrust in the value of conventional tumor-staging nomenclature in terms of reliably predicting the risk of tumor relapse even in patients with early pancreatic cancer (T_1, N_0). Our data indicate that immunohistochemical assessment of lymph nodes can be used to refine the staging system for pancreatic ductal adenocarcinoma and might help us to identify patients who could not be cured by surgery alone and need adjuvant therapy. In turn, patients who are true node-negative both in histopathology and in immunohistochemistry have an excellent five-year survival probability of nearly 60%, even without chemotherapy. Whether this prognosis can be further improved by adjuvant therapy needs further study.

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