Pancreatic Lymph Nodal and Plexus Micrometastases Detected by Enriched Polymerase Chain Reaction and Nonradioisotopic Single-Strand Conformation Polymorphism Analysis: A New Predictive Factor for Recurrent Pancreatic Carcinoma

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ABSTRACT

K-ras point mutations have been observed in approximately 90% of pancreatic carcinomas. We genetically analyzed cases of pancreatic regional lymph nodal and plexus micrometastases in invasive ductal carcinoma of the pancreas who were node negative or had metastases limited histopathologically to pancreaticoduodenal lymph nodes. These cases underwent curative resection in our institute. The utility of genetic analysis was compared with that of histopathological study, in terms of postoperative clinical outcome, as a predictive factor for recurrent pancreatic carcinoma.

Samples for DNA extraction were obtained from formalin-fixed, paraffin-embedded specimens. A 0.5-μg quantity of DNA was subjected to enriched PCR and nonradioisotopic single-strand conformation polymorphism analysis. K-ras codon 12 mutations were detected in 83% (10 of 12) of invasive ductal carcinomas. In four cases, the genetic analysis of regional lymph nodal metastases and pancreatic plexus invasion of the pancreatic carcinoma yielded results concordant with those of histopathological analysis. In six cases, however, the metastases detected by genetic analysis were more advanced than was indicated by the histopathological examination. The survival rate of cases with metastases beyond the pancreaticoduodenal lymph nodes was significantly lower than that of cases with metastases limited to the pancreaticoduodenal lymph nodes or with no nodal involvement based on genetic analysis (P < 0.05).

Intraoperative analysis of point mutations at K-ras codon 12 in the regional lymph nodes and the pancreatic plexus by enriched PCR/nonradioisotopic single-strand conformation polymorphism analysis is a highly accurate predictive factor for recurrent pancreatic carcinoma.

INTRODUCTION

The incidence of adenocarcinoma of the pancreas has been increasing worldwide in recent years (1, 2). It is currently the fourth leading cause of cancer-related mortality in North America (1) and the fifth leading cause in Japan (2). In Japan, there were more than 14,000 pancreatic cancer deaths in 1992, and the mortality rate has since been rising annually (3). Definitive curative resection was possible in no more than approximately 10% of all cases (3). This low rate is attributable to difficulties diagnosing pancreatic adenocarcinoma at an early stage. Owing to advances in diagnostic modalities, the rate of detection for small pancreatic adenocarcinomas has increased (3). Small pancreatic carcinomas, particularly in node-negative cases, were expected to have a good prognosis (4). However, even among curatively resected pancreatic adenocarcinomas, including small, node-negative tumors resected at an early stage, there are typically several relapses resulting in death within a short period. Major sites of recurrence include the pancreatic bed, regional lymph nodes, the liver, and peritoneal surfaces (5). Only a small number of curatively resected cases enjoy prolonged survival. The differences between cases with long-term and short-term survival cannot be fully accounted for even by detailed histopathological study.

In a genetic analysis of colorectal cancer, Hayashi et al. (6) demonstrated that a small number of cancer cells had already spread into several lymph nodes by the time of tumor resection. Lymph nodal metastasis had not been diagnosed histopathologically in their cases. Reportedly, genetically detected lymph nodal metastasis shows a good correlation with clinical outcome (7). Genetically diagnosed lymph nodal metastasis may be a useful prognostic factor in colorectal cancer.
K-ras mutations located primarily at codon 12 have been observed in approximately 90% of pancreatic carcinomas (8–11). We thus hypothesized that genetic detection of micrometastases might be a more useful predictive factor for patients with pancreatic carcinoma than for those with colorectal cancer.

In the present study, we retrospectively analyzed K-ras codon 12 point mutations in regional lymph nodes and the pancreatic plexus from specimens from pancreatic invasive ductal carcinomas using enriched PCR (12, 13) and non-RI SSCP analysis (14, 15). The analyses were conducted under the assumption that patients with no regional nodal metastasis and/or metastasis limited to pancreaticoduodenal lymph nodes, as demonstrated by genetic analysis, would have a good prognosis. We analyzed not only node-negative cases but also those with lymph nodal metastases limited to the anterior and posterior pancreaticoduodenal area because the latter show approximately same survival rate as node-negative cases (16).

PATIENTS AND METHODS

Inclusion Criteria. Pancreatic carcinoma cases were included in this study if the tumor was located at the head of the pancreas, if it was 3.0 cm or less in diameter, if it was limited to the pancreas or extended no further than the bile duct or duodenum, if lymph nodes were negative or nodal metastasis was limited to the pancreaticoduodenal lymph nodes based on histopathological study, and if patients had undergone definitive curative resection of an invasive ductal carcinoma. Curative resection was defined as complete removal of the tumor with a histologically clear margin at the bile duct and pancreas, as well as in the peripancreatic soft tissue, along with removal of metastases involving the primary and secondary lymph node groups. This definition was based on the Classification of Pancreatic Carcinoma established by the Japan Pancreas Society (17). Twenty-two patients who had been operated on and treated at Keio University Hospital (Tokyo, Japan) from 1980 through 1991 met these criteria. All specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Histological diagnoses were made by one experienced pathologist (M. M.) according to the Classification of Pancreatic Carcinoma established by the Japan Pancreas Society criteria (17). Twelve of 22 DNA samples were preserved in good condition, allowing amplification by enriched PCR, and were thus suitable for the study.

Cell Lines. The human stomach carcinoma cell line MKN-1 was obtained from the Japanese Cancer Research Resource Bank (14). The human pancreatic tumor cell line PSN-1 was provided by the Division of Pathology, National Cancer Center Research Institute (Tokyo, Japan; Ref. 18).

DNA Extraction. The paraffin blocks were cut into one 4-μm and several 20-μm sections. The 4-μm section was stained with H&E for histopathological diagnosis to differentiate neoplastic from normal tissues. DNA samples were extracted from unstained sections of the malignant tissue, lymph nodes, and the pancreatic plexus samples. DNA was extracted from each specimen by treatment with SDS-proteinase K and phe-
with a cooling pipe in which cool (14–16°C) water circulated continuously. The running time was 4 h. The gel was then silver stained using a kit purchased from Daiichi Pure Chemical Company, Ltd. (Tokyo, Japan) according to the manufacturer’s instructions.

Statistical Analysis. The cumulative survival rates for patient groups were calculated by the Kaplan-Meier method and compared using the generalized Wilcoxon test.

RESULTS

Sensitivity of the Analysis. One-μg quantities of the DNA extracted from normal peripheral blood lymphocytes and a pancreas carcinoma cell line were subjected to PCR. The sensitivity of the analysis was sufficient to detect 0.1% of mutant K-ras DNA after the second PCR (data not shown).

K-ras Analysis of Tumor Tissue. A total of 12 pancreatic carcinomas were studied. Ten of the 12 had point mutations at K-ras codon 12 in the tumor tissue (83%). The two tumors that did not have point mutations at K-ras codon 12 did not differ from the 10 carrying these point mutations in terms of clinicopathological characteristics.

Histopathological Study. All 10 tumors with K-ras mutations were invasive ductal carcinomas. Six were node negative and four had metastases limited to the anterior and/or posterior pancreaticoduodenal lymph nodes. With the exception of one case, no invasion of the pancreatic plexus was observed. Clinical data from the 10 pancreatic carcinoma patients are summarized in Table 1. There was no significant difference in the cumulative survival rate, based on statistical analysis, between the node-negative cases and those with lymph nodal metastases limited to the anterior and posterior pancreaticoduodenal lymph nodes, as demonstrated by histopathological study. The mean follow-up period of these 10 cases was 45.8 months, and the median was 26 months (range, 8–133 months). Pathological examinations were performed according to the methods proposed by the Japan Pancreatic Society (17).

K-ras Analysis of Lymph Nodes and Pancreatic Plexus. We analyzed lymph nodes and the pancreatic plexus in the 10 cases carrying K-ras codon 12 point mutations at the primary tumor.

Lymph nodes and the pancreatic plexus were classified according to the Classification of Pancreatic Carcinoma established by the Japan Pancreas Society (Fig. 1; Ref. 20). When the K-ras mutation pattern in a lymph node or the plexus demonstrated by non-RI SSCP was the same as that of tumor, the lymph node or the plexus was considered to be positive for metastasis (Fig. 2). The results of genetic analysis were com-

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### Table 1 Patient characteristics

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age</th>
<th>Primary tumor</th>
<th>Greatest dimension (cm)</th>
<th>Surgical procedure</th>
<th>Stage</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>69</td>
<td>T2(bd)</td>
<td>2.8</td>
<td>PPPD'</td>
<td>I</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>60</td>
<td>T2(du)</td>
<td>3.0</td>
<td>PD</td>
<td>I</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>54</td>
<td>T1b</td>
<td>2.5</td>
<td>TP</td>
<td>I</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>65</td>
<td>T2(bd)</td>
<td>2.7</td>
<td>PD</td>
<td>I</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>55</td>
<td>T1a</td>
<td>1.5</td>
<td>PD</td>
<td>I</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>55</td>
<td>T1b</td>
<td>2.8</td>
<td>PD</td>
<td>I</td>
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<td>7</td>
<td>Male</td>
<td>40</td>
<td>T1b</td>
<td>2.5</td>
<td>PD</td>
<td>III</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>69</td>
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<td>PD</td>
<td>III</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>63</td>
<td>T1a</td>
<td>2.0</td>
<td>PD</td>
<td>III</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>57</td>
<td>T2(du)</td>
<td>3.0</td>
<td>PD</td>
<td>III</td>
</tr>
</tbody>
</table>

* T category proposed by the UICC: T2(bd), tumor extends bile duct; T2(du), tumor extends duodenum.

* Stage grouping proposed by the UICC: TNM classification of malignant tumors (29).

* PPPD, pylorus-preserving pancreaticoduodenectomy; PD, pancreaticoduodenectomy; TP, total pancreatectomy.
Fig. 2 Detection of point mutations of the K-ras gene in the primary tumor, lymph nodes, and plexus by non-RI SSCP analysis. a, DNA derived from the primary tumors. Tumors J and L had no point mutations at K-ras codon 12. The other 10 tumors had point mutations at K-ras codon 12 (arrowheads). b, Lanes 1-5. DNAs were derived from lymph nodes and plexuses of the same case. The band of the primary tumor is shown in Lane D in a. When lymph nodes and plexuses were examined, DNAs obtained from the primary tumor, pancreatic cancer cell line (PSN1), and stomach cancer cell line (MKN1) were loaded on the same gel. Lanes 1 and 5 represent mutated K-ras and suggest the presence of metastasis because the pattern detected by non-RI SSCP was the same as that of the primary tumor. The metastatic lymph node or plexus tissues in b contain normal cells together with cancer cells in the tissue. The proportion of cancer cells is different between the lymph nodes or plexuses. Consequently, the metastatic tissue shows mutant bands with high intensity and wild-type bands in SSCP analysis. On the other hand, primary tumor tissues are rich in tumor cellularity, thus, the band of normal DNA is faint. Lymph node and plexus tissues with metastasis usually show a mixture of bands derived from mutated and wild-type alleles.

pared with histopathological results as shown in Fig. 3. The precise locations of lymph nodal metastases and the postoperative status of these 10 cases are shown in Table 2. According to the results of the genetic analysis, the patients were divided into two groups: (a) node-negative cases and those with nodal metastasis limited to pancreaticoduodenal lymph nodes; and (b) cases with metastasis beyond the pancreaticoduodenal lymph nodes based on genetic analysis. The 5-year survival rates were 60 and 0%, respectively. The survival rate of cases with lymph nodal metastasis beyond the pancreaticoduodenal area, as demonstrated by genetic analysis, was significantly lower than that of the cases with no metastasis or only limited metastasis ($P < 0.05$; Fig. 4).

**DISCUSSION**

In this series, 10 of 12 pancreatic tumors had point mutations of the K-ras oncogene at codon 12 (83%). Hruban et al. (11), who examined a large number of pancreatic tumor specimens ($n = 450$), reported that 85% of pancreatic adenocarcinomas had point mutations at K-ras codon 12. The frequency of point mutations at K-ras codon 12 among our 12 cases is thus highly consistent with those of previous reports (8–11).

Various sensitive and relatively simple methods of detecting point mutations in ras oncogenes have been reported (6–7, 12–15, 21–23). Among these methods, non-RI SSCP analysis yields sharp bands as the silver stain used can detect slight migration, even in a small slab gel, such that specific patterns can be visualized directly in each case (14). This simplifies the process of confirming whether or not the mutations detected are the same. In the present study, when the same mutations to the primary tumor were found in regional lymph nodes or the plexus, those were judged to be positive for metastasis. Because the electrophoretic profile is specific to each mutation, identification of a specific mutation is possible by comparing the mobilities of the sample DNAs with those of control DNA carrying a known mutation. This method reportedly also yields highly reproducible results (15, 24).

The metastatic lymph node or plexus tissues shown in Fig. 2B contain normal cells together with cancer cells in the tissue. The proportion of cancer cells is different between each lymph
Because before 1990, it was not known that samples would be used for genetic study. Furthermore, samples were taken from 1980 through 1991; in the case of the oldest sample, DNA was extracted 16 years after they were obtained. Almost all samples obtained after 1990 could be amplified.

Perineural invasion occurred in at least 90% of cases with invasive ductal carcinoma of the pancreas. Invasion to the pancreatic plexus is one of the important factors determining the extent of pancreatic carcinoma. Thus, it is very important to examine not only regional lymph nodes but also the pancreatic plexus in using this approach to pancreatic carcinoma. Two of our cases, patients 5 and 9, had pancreatic plexus invasion as revealed by genetic analysis, although in patient 9 it was also detected histopathologically. These two cases died within 3 years.

Small or node-negative pancreatic carcinomas [in particular, those with tumors less than 2 cm in diameter (4)] are generally considered to have a relatively good prognosis. However, those with the a tumor size of 2–3 cm show relatively prolonged survival compared with those with tumors larger than 3 cm. In our institute, the 3- and 5-year survival rates for of the carcinoma of the pancreas head were 2.4 and 2.4% (tumors larger than 3 cm), 26 and 10% (tumors 2–3 cm), and 40 and 30% (tumors smaller than 2 cm), respectively. Because only 2.4% of 5-year survivors had a tumor size greater than 3 cm, we excluded patients with tumors larger than 3 cm.

Moreover, node-negative pancreatic carcinoma cases are widely regarded as showing prolonged survival. Cameron et al. (27) reported that the strongest factor influencing long-term survival in cancer at the head of the pancreas was lymph node status. However, recurrences are seen in some node-negative cases shortly after curative resection. On the other hand, cases with metastasis limited to lymph nodes in proximity to the carcinoma show prolonged survival. Patients with carcinoma of the pancreatic head in whom metastases are limited to the anterior or posterior pancreatoduodenal lymph nodes have a survival rate approaching that of node-negative cases (16). Therefore, we analyzed not only node-negative cases but also those with lymph nodal metastasis limited to the pancreatoduodenal area, according to histopathological examination. Furthermore, the results of our genetic analysis confirmed that cases with metastases limited to the pancreatoduodenal lymph nodes show prolonged survival. In our present series, patients 7 and 8 have been alive and recurrence free for 8 and 11 years, respectively. Both are 5-year survivors of pancreatic carcinoma. Patients 2 and 3 died of heart failure and suffocation, respectively. Autopsy confirmed both to be free of carcinoma recurrence. One of our cases that were node negative and in which nodal metastasis was limited to pancreatoduodenal lymph nodes died of his pancreatic malignancy after a 4 year-prolonged survival. Liver metastases and local recurrence were observed in patient 1, despite node-negative status according to histopathological and genetic study. Local recurrences have two components, lymph node and pancreatic bed recurrence. Distinguishing between these two can be difficult.

All cases underwent curative resection. In our institute, the 5-year survival rate of carcinomas of the pancreatic head that underwent curative resection was 21.3% (n = 61; Ref. 28). The 5-year survival rate of carcinomas of the pancreatic head less

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**Fig. 3** A, on the basis of genetic analysis results, histopathologically node-negative cases (n = 6) were divided into three groups: node negative, lymph nodal metastases limited to the pancreatoduodenal area, and metastases beyond the pancreatoduodenal area. The groups contained 2, 1, and 3 cases, respectively. B, four cases with lymph nodal metastases limited to the pancreatoduodenal area, histopathologically, were divided into two groups based on genetic analysis; two cases had lymph nodal metastases limited to the pancreatoduodenal area, whereas the other two had metastases beyond the pancreatoduodenal area.

node or plexus. Consequently, the metastatic tissue shows mutant bands with high intensity and wild-type bands in SSCP analysis. On the other hand, primary tumor tissues are rich in tumor cellularity; thus, the band of normal DNA is faint. Lymph node and plexus tissues with metastasis usually show a mixture of bands derived from mutated and wild-type alleles. Furthermore, there is a band above the mutant bands in Lane D of bands derived from mutated and wild-type alleles. Further...
Table 2 Discrepancy between histopathological and genetic analysis results for lymph nodal and plexus metastases

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Histopathological analysis</th>
<th>Genetic analysis</th>
<th>Status</th>
<th>Cause of death</th>
<th>Duration of follow-up (months)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0/12 n0</td>
<td>0/12 n0</td>
<td>DOD</td>
<td>Li, Lo</td>
<td>48</td>
</tr>
<tr>
<td>2</td>
<td>0/51 n0</td>
<td>0/51 n0</td>
<td>NED</td>
<td>Heart failure</td>
<td>82</td>
</tr>
<tr>
<td>3</td>
<td>0/85 n0</td>
<td>4/85 n1, n0, 13</td>
<td>NED</td>
<td>Suffocation</td>
<td>26</td>
</tr>
<tr>
<td>4</td>
<td>0/27 n0</td>
<td>5/27 n1, n0, 17, 12</td>
<td>DOD</td>
<td>Lo, Li</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>0/29 n0</td>
<td>5/29 n1, 8, PL sma</td>
<td>DOD</td>
<td>Li, Lo, P</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>0/26 n0</td>
<td>5/26 n1, n0, 16</td>
<td>NED</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>7</td>
<td>1/49 n1, n0, 13</td>
<td>1/49 n1, n0, 13</td>
<td>NED</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>8</td>
<td>2/56 n1, n0, 17</td>
<td>2/56 n1, n0, 17</td>
<td>NED</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>4/21 n1, n0, 13, 17, PL phII</td>
<td>9/21 n1, n0, 13, 17, 12, 16, PL phII</td>
<td>DOD</td>
<td>Lo, P</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>2/19 n1, n0, 13, 17</td>
<td>4/19 n1, n0, 13, 17, 12, 16</td>
<td>DOD</td>
<td>Lo, Bo</td>
<td>8</td>
</tr>
</tbody>
</table>

* NOM, no. of metastases (positive/examined); LOM, location of metastases; DOD, died of disease (primary malignancy); NED, no evidence of disease (no recurrence); Li, liver metastasis; Lo, local recurrence; P, peritonitis carcinomatosa; Bo, bone metastasis.

Fig. 4 Cumulative survival curve for node-negative patients or those with metastases limited to the lymph nodes in the pancreaticoduodenal region (line a) and the curve for patients with lymph node metastases beyond the pancreaticoduodenal region (line b), based on genetic analysis, as calculated by the Kaplan-Meier method. The survival rates of these two groups differed significantly (P < 0.05). *, P = 0.012.

than 2 cm in maximum diameter was 37% (4). These 5-year survivors underwent clinically curative resection; in other words, tumors and micrometastases were completely removed. In our present study, 5-year survival rate of 10 cases was 30%. Patients 3, 7, and 8 had metastases involving the anterior and/or posterior pancreaticoduodenal lymph nodes based on genetic analysis. Patients 7 and 8, however, have been recurrence free for more than 5 years. Patient 3, who died of suffocation, was recurrence free for 26 months. The outcomes of the patients suggest that all lymph nodal micrometastases were completely removed in these three patients.

Patient 3 underwent total pancreatectomy because of intraductal growth of the carcinoma. Radiation therapy was administered to patients 4–10. Patients 4, 5, 7, and 8 received extra-corporeal radiation after surgery (total dose, 25 Gy), whereas patients 6, 9, and 10 received intraoperative radiotherapy (20 Gy, 6 MeV). Patients 4, 5, 8, 9, and 10 received chemotherapy in the form of continuous intraportal injection of 250 mg of 5-fluourouracil per day for 7 days starting on the day of surgery. Adjuvant therapy differed among cases. However, the majority of previous studies have suggested that neither intraoperative radiotherapy alone nor intraoperative radiotherapy with chemotherapy achieves long-term survival.

All cases with nodal metastases beyond the pancreaticoduodenal lymph nodes, as demonstrated by genetic analysis, died within 3 years due to local recurrence or other types of recurrence (e.g., hepatic, bone, or peritoneal surface metastasis). These may even have been advanced cases erroneously diagnosed as early stage pancreatic carcinoma by histopathological examination. Histopathological examinations could be performed on samples that were only 4 μm thick. However, our genetic examination method requires sections that are more than 200 μm thick. Thus, genetic examinations must be performed on samples approximately 50 times as large as those used in routine histopathological study.

It is not certain whether the mutations detected by enriched PCR reflect the presence of viable tumor cells in the nodes. It is reasonable to assume that nonviable tumor cells were ingested by phagocytes and thereby were transported to exist in lymph nodes. However, our genetic analysis results reflect clinical outcomes more accurately than histopathological findings, just as in case of colon cancer as reported by Hayashi et al. (6, 7).

There are three sites that are often associated with treatment failure in pancreatic carcinoma: the pancreatic bed and regional lymph nodes, the liver, and peritoneal surfaces (5). Focusing on these sites is very useful for predicting local recurrence. However, reliable means of predicting hepatic metastases and peritoneal implantation have yet to be developed. At advanced clinical stages, the incidences of hepatic metastases and peritoneal implantation rise. Predicting the survival period of pancreatic carcinoma in far advanced cases requires evaluation based on the three aforementioned components. We cur-
rently investigating genetic predictive factors for the hepatic metastases of pancreatic carcinoma.

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Pancreatic lymph nodal and plexus micrometastases detected by enriched polymerase chain reaction and nonradioisotopic single-strand conformation polymorphism analysis: a new predictive factor for recurrent pancreatic carcinoma.

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