Detection of K-ras Gene Mutations in Plasma DNA of Patients with Pancreatic Adenocarcinoma: Correlation with Clinicopathological Features

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ABSTRACT

We investigated the presence of K-ras gene mutation in plasma DNA and assessed its clinical value in patients with pancreatic adenocarcinoma. Mutations in codon 12 of the K-ras gene were examined by mutant allele-specific amplification method using DNA extracted from surgical specimens and plasma samples of 21 patients with pancreatic adenocarcinoma. K-ras gene mutation was detected in 15 of 21 (71%) primary tumors. In 9 of 15 (60%) patients with K-ras gene mutation-positive tumors, an identical mutation was detected in the plasma DNA. None of four patients with chronic pancreatitis or five healthy subjects had such mutations in plasma DNA. Tumors positive for K-ras gene mutation in plasma DNA were significantly larger (P = 0.04) and less likely to result in a curative cure after surgical resection (P = 0.09) than those negative for the mutation. Other clinicopathological features, including age, sex, histological type, mode of invasion, and metastasis, did not correlate with K-ras gene mutations in plasma DNA. Treatment resulted in disappearance of K-ras gene mutations in plasma DNA in six of nine (67%) patients. Three patients with a persistently positive K-ras gene mutation in pre- and post-treatment plasma samples were likely to show early recurrence or have a progressive disease. Our findings suggest that K-ras gene mutation can be detected in plasma DNA of patients with pancreatic adenocarcinoma. Detection of K-ras mutations in plasma may be clinically useful for evaluating tumor burden and efficacy of treatment.

INTRODUCTION

The incidence of pancreatic adenocarcinoma has increased steadily in recent years and is, at present, the fourth and fifth leading cause of cancer death in men and women, respectively (1). Despite recent advances in diagnosis and treatment, pancreatic adenocarcinoma remains one of the most distressing diseases, both for the patient and the physician (2, 3). Surgical resection is the only treatment currently offering these patients an opportunity for longer survival and cure. However, despite the recent approach of aggressive surgical resection of the tumor, about two-thirds to one-half of the patients still do not live more than 5 years after such surgery (4–9).

Because the tumor is frequently beyond surgical resectability or has already metastasized when symptoms first appear, the resectability rate remains ≤20% (10). Palliative treatment may, in some cases, offer less morbidity and reduces hospital time and expenses (10–13). Furthermore, the value of surgical treatment in those patients who are thought to have potentially resectable pancreatic adenocarcinoma has even been questioned. The need to appropriately identify and treat only those patients who have a substantial chance for curative resection of the tumor and long-term survival after treatment have been emphasized (10–14). Therefore, a reliable and sensitive method for accurate evaluation of the extent of local and distant spread of pancreatic cancer should improve prognosis and, hence, provide a better planning and optimal management strategies to improve the quality of life (3, 10).

Recent efforts to predict those patients who would most benefit from radical resection have identified the size of tumor, spread to lymph nodes, extent of invasion to blood vessel and retroperitoneal space, and histological grade as important factors that influence survival after resection (4, 5, 7, 8, 15–20). However, such information is obtained by clinical examination or pathological evaluation and is not always accurate in predicting the surgical outcome, especially within the same stage.

Recent studies using PCR and a variety of tumor markers have identified mutations of K-ras gene in certain tumors (21). In particular, point mutations of K-ras gene are present in 70–100% of pancreatic adenocarcinomas (22–25), as well as in certain precancerous lesions such as ductal hyperplasia (26). This point mutation has been successfully used for screening or diagnosis of pancreatic adenocarcinomas using samples of pancreatic juice, blood, stool, or aspirated pancreatic tissue (27–29).

Recent studies have demonstrated that detection of genetic alterations affecting plasma or serum DNA is now a reliable method for identifying a variety of malignancies such as lung (30), head and neck (31), and colorectal (32) cancers. Here, we examined the presence of K-ras gene mutation in plasma DNA.
The mean age of patients with pancreatic adenocarcinoma was 63.9 years (range = 35–79 years; 13 males and 8 females). The surgical treatment was considered to have produced a successful cure of the condition in seven patients with pancreatic adenocarcinoma, based on clinical and pathological findings (R0, resection according to International Union Against Cancer TNM3 classification; Ref. 33). The other 14 patients with pancreatic adenocarcinoma underwent R1,2 resection, bypass operation, or simple laparotomy, followed by radio- or chemotherapy. In this study, adequate tumor tissue was available from each patient for pathological examination and molecular genetic analysis. Tissue specimens for molecular analysis were immediately frozen at surgery and kept at −80°C until use. All 21 tumors were macro- and microscopically examined to determine location, size, extent and mode of invasion, and metastasis to lymph nodes or distant organs. Sixteen tumors were located in the head of the pancreas, and five were in the body or tail. The tumors were macro- and microscopically examined to determine location, size, extent and mode of invasion, and metastasis to lymph nodes or distant organs. Sixteen tumors were located in the head of the pancreas, and five were in the body or tail. The mean size of the tumor was 3.9 cm in diameter, ranging from 1.5 to 9.0 cm. The disease stage was classified according to the international TNM staging system (33): 2 patients with stage I, 3 with stage II, 3 with stage III, and 13 patients with stage IV.

All patients were thoroughly examined at follow-up, including laboratory tests (routine blood count and levels of serum CA19-9) at 1-month intervals, ultrasound examination, X-ray of the chest, and computerized tomographic scan at 3-month intervals. Follow-up care was organized by Department of Surgery II, and all patients were followed until death or the end of the observation period (December 31, 1997). The follow-up period ranged from 1.3 to 43.5 months (mean = 12.2 ± 9.6 months).

Plasma and Tissue DNA. Blood samples (10 ml) were withdrawn from a peripheral vein and placed in tubes containing sodium citrate for plasma extraction. The collected sample was divided into two parts. The first part, used for plasma DNA extraction, was centrifuged at 4°C for 30 min at 1000 × g, and the supernatant was stored at −80°C until use. The other part was used for isolation of mononuclear cells and was processed immediately by centrifugation with Ficoll-Paque (Pharmacia, Uppsala, Sweden) after addition of 2% dextran and agitation of RBCs. To deplete residual RBCs, the deposits were washed in 0.2% NaCl, and to deplete residual plasma components, mononuclear cells, e.g., lymphocytes, were washed in PBS at least three times and frozen until use.

Tumor tissues were obtained at surgery and immediately cut into two sections. The first was fixed in 10% buffered formaldehyde for histopathological diagnosis. The remaining half was immediately frozen and stored at −80°C until DNA extraction for molecular analysis.

Plasma, tissue specimen, and mononuclear cells for DNA extraction were digested in SDS and proteinase K at 56°C overnight, followed by phenol and chloroform extraction and then isopropyl alcohol precipitation of DNA, as described previously (34).

Detection of K-ras Gene Mutation. Mutation at codon 12 of the K-ras gene was examined by MASA method, as described previously, with slight modifications (35). Briefly, 100 ng of DNA were used as a PCR template in a 25-µl reaction mixture. PCR with wild-type primers (Table 1) was performed using one cycle each for 3 min at 94°C, 2 min at 62°C, and 3 min at 72°C, followed by 39 cycles for 1 min at 94°C, 1.5 min at 62°C, and 1 min at 72°C, with a final extension step of 72°C for 10 min. For PCR with MASA primers, the 3’-ends of 20-bp oligonucleotides used as PCR primers corresponded to variants of the first or second nucleotide in codon 12 of the K-ras gene (Table 1). PCR with MASA primers was performed using the same protocol for wild-type primers, except that the annealing temperature was 65°C. PCR products were electrophoresed in 2% agarose gel containing 0.5 µg/ml ethidium bromide with a size maker. The sample was considered positive for mutation of K-ras gene when PCR products were recognized as a fluorescent band under UV after electrophoresis. The study was performed in a blinded fashion, such that the patients’ clinical details and histopathology were unknown to the investigator performing the MASA assay.

Statistical Analysis of Survival Curves. Statistical comparisons of baseline data between groups were tested by χ2
Fig. 1  Ethidium bromide staining of MASA-PCR products. MASA-PCR products were electrophoresed in 2% agarose gels containing 1 μg/ml ethidium bromide. Lane M, size marker (1-kb DNA ladder); Lane NC, negative control; Lanes PC, positive controls; Lanes 1–4, DNA from the primary tumor of a representative patient with pancreatic adenocarcinoma (Lane 1, first-letter mutant; Lane 2, second-letter mutant; Lane 3, mutant in both letters; Lane 4, nonmutant); Lanes 1′–4′. DNA from plasma of the same patients in Lanes 1–4, respectively. Note that each product from plasma DNA has a pattern similar to that of the primary tumor except for Lane 3′, which shows only a second-letter mutant instead of both-letter mutations that were detected in the primary tumor.

test or Student’s t test. The difference was considered significant when \( P < 0.05 \).

**RESULTS**

**K-ras Gene Mutation in Primary Tumor of Pancreatic Adenocarcinoma.** K-ras gene mutations were recognized as fluorescent bands under UV after electrophoresis, and the product size was 180 bp for the wild-type primer and set 1 primers for the first-letter mutations and 179 bp for set 2 primers for the second-letter mutations (Fig. 1). The wild type K-ras gene was detected in all primary tumors. Fifteen (71%) of 21 primary tumors of pancreatic adenocarcinoma had mutations in the first and/or the second letter of codon 12 in K-ras gene, whereas none of the pancreatic tissues from patients with chronic pancreatitis contained mutations. Among 15 tumors with K-ras gene mutations, 6 (40%) had mutation at the first letter, and 11 (73%) had mutation at the second letter. Two patients demonstrated K-ras mutations in both letters. No significant difference in clinicopathological findings, including age, sex, location, histological type, mode of invasion and metastasis, tumor stage, curative tumor resectability, and clinical outcome, were found between patients with pancreatic adenocarcinomas positive or negative for K-ras gene mutation (Table 2).

**K-ras Gene Mutations in Plasma and Peripheral Lymphocytes.** Nine (60%) of 15 patients whose primary tumors had K-ras mutations showed identical mutations in plasma DNA prior to any treatment (Fig. 2), which were confirmed by direct sequencing (data not shown). One patient in whom K-ras gene mutations in both the first and second letter of the codon 12 were detected in the pancreatic tumor showed only the second-letter mutation in plasma DNA. No mutations were detected in plasma DNA of six patients with pancreatic adenocarcinoma who were negative for K-ras gene mutations. Furthermore, no mutations were detected in plasma DNA of patients with chronic pancreatitis or normal subjects. No mutations were detected in DNA from the mononuclear cells of all subjects.

**Association of K-ras Mutations in Plasma DNA with Clinicopathological Features.** To identify factors associated with the presence of K-ras mutations in plasma DNA, we examined the clinicopathological features of 15 patients with K-ras mutations in primary tumors. These patients were divided into two groups: K-ras mutation positive in plasma DNA and K-ras mutation negative in plasma DNA. Patients with K-ras gene mutation in plasma DNA obtained preoperatively were more likely to have large tumors than those patients who were negative for K-ras gene mutation in plasma DNA (\( P = 0.04 \)), and surgery was less likely to result in a curative cure (\( P = 0.09 \)) in those negative for K-ras gene mutation in plasma DNA (Table 3). However, there were no statistical differences between plasma DNA K-ras-positive and -negative groups in age, sex, histological type, tumor invasiveness (T factor), lymph node metastasis (N factor), distant metastasis (M factor), or stage (Table 3).

**Detection of Mutant DNA in Plasma after Treatment.** The presence of K-ras gene mutations in plasma DNA was restated 1–2 months after treatment in all patients with mutant DNA detected in primary tumor (Table 4). The mutant DNA in plasma disappeared in four patients who underwent curative or palliative tumor resection and another two patients who were treated with an intensive course of chemotherapy and radiotherapy, whereas they were positive for K-ras mutation in pretreatment plasma DNA. In three patients, however, K-ras gene mutation in plasma DNA was detected persistently after treatment. Among these three patients, one was treated by macro- and microscopically curative resection, and the other two were treated by...
Circulating K-ras mutation in pancreatic adenocarcinoma

Chemo-/radiotherapy. Especially, one patient (patient 9 in Table 4) who was persistently positive for K-ras mutation in plasma DNA recurred 6 months after operation and died within 1 year, despite the tumor’s early stage and macro- and microscopically curative resection. Furthermore, patients with persistent K-ras-positive plasma DNA were likely to have a poorer prognosis than the patients who were negative for K-ras mutations in plasma DNA of posttreatments (mean survival = 9 versus 13 months). The disappearance of the mutant DNA in plasma was, however, not always associated with a change in serum concentrations of CA19-9, a useful marker for clinical outcome of patients with pancreatic adenocarcinoma (36).

**DISCUSSION**

High serum or plasma levels of DNA are present in patients with various malignancies (34, 37-39). These studies suggested that DNA derived from the primary tumor might appear in plasma and that increased circulating tumor DNA in the bloodstream but not in the cancer cells themselves resulted in high serum or plasma DNA levels. They also indicated that high serum or plasma DNA levels are associated with advanced stage tumors and that effective therapy may reduce these levels. Recent studies using gene alterations as tumor-specific makers clearly demonstrated that gene alterations corresponding to tumor DNA are detected in the serum or plasma and suggested the potential usefulness of the presence of such gene alterations in the serum or plasma for staging, management, and detection of tumors (30-32, 40). Recent studies using genetic analysis of adenocarcinoma have shown frequent DNA mutations of the K-ras gene in codon 12, 13, or 61 (21). In particular, K-ras gene mutations are found in 70–100% of pancreatic adenocarcinoma, and most mutations occur in codon 12 (22-29). These mutations have been used clinically for the diagnosis of pancreatic adenocarcinoma (27-29). In this study, we examined tumor tissue and plasma DNA samples for K-ras gene mutations in codon 12 to evaluate the clinical usefulness of detection of such mutations on tumor staging and efficacy of treatment.

Previous studies have reported the presence of K-ras gene mutations in circulating DNA extracted from plasma or serum samples of patients with colorectal (32) and pancreatic cancers (40) and leukemia (41). However, the clinical value of detection of such mutations in the plasma remains obscure due to either a lack of comparison with clinicopathological variables or the short follow-up period in these studies. Our results showed that mutant K-ras DNA was present in the plasma of patients with pancreatic adenocarcinoma and suggested that monitoring such mutation in plasma DNA may be clinically useful.

Table 3 Differences in clinicopathological features of pancreatic ductal adenocarcinomas with mutation of K-ras gene associated or not associated with mutations of the same gene in plasma DNA

<table>
<thead>
<tr>
<th>Variable</th>
<th>Positive (n = 9)</th>
<th>Negative (n = 6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr, mean ± SD)</td>
<td>62.6 ± 12.1</td>
<td>64.7 ± 6.2</td>
<td>0.70</td>
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<tr>
<td>Sex (M/F)</td>
<td>7/2</td>
<td>3/3</td>
<td>0.26</td>
</tr>
<tr>
<td>Histological type (well/moderate)</td>
<td>7/2</td>
<td>5/1</td>
<td>0.79</td>
</tr>
<tr>
<td>Tumor size (cm, mean ± SD)</td>
<td>4.2 ± 1.5</td>
<td>2.6 ± 0.9</td>
<td>0.04</td>
</tr>
<tr>
<td>TNM Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T (1-3/4)</td>
<td>7/2</td>
<td>6/0</td>
<td>0.83</td>
</tr>
<tr>
<td>N (0/1)</td>
<td>6/3</td>
<td>4/2</td>
<td>1.00</td>
</tr>
<tr>
<td>M (0/1)</td>
<td>5/4</td>
<td>4/2</td>
<td>0.16</td>
</tr>
<tr>
<td>Tumor stage (I–III/IV)</td>
<td>3/6</td>
<td>3/3</td>
<td>0.52</td>
</tr>
<tr>
<td>Resectability (curative/palliative or nonresected)</td>
<td>2/7</td>
<td>4/2</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*Well, well-differentiated adenocarcinoma; moderate, moderately differentiated adenocarcinoma; curative, curative resection; palliative, palliative resection; nonresected, not resected.

Although the presence of cancer DNA in plasma or serum has been recently described (30-32, 40), the underlying process that causes cancer DNA to be released into circulation has yet to be explained and remains a controversial issue. One possible mechanism includes cell lysis, resulting from physical and immunological damage, because cancer cells are immunologically foreign to their hosts and have an extensive vascular interface with them. If the tumors have a more broad tumor burden, the tumor cells could have more damage and release more free DNA into plasma. The finding that patients with mutations in plasma were more likely to have large tumors and were less likely to have curative resection than those who were negative for mutations in plasma DNA. Furthermore, patients who were persistently positive for mutations in plasma obtained after treatment were likely to develop early recurrence or to have poor prognosis.
adenocarcinoma; moderate, moderately differentiated adenocarcinoma: NED, no evidence of disease; DOD, death due to pancreatic adenocarcinoma:

K-ras

attachment of serum CA19-9 level with positivity of creatine cancer (46). We did not always observe the close asso-

Serum CA19-9 outcome of patients with pancreatic adenocareinoma (36),

K-ras mutation in plasma. In those patients, detection of plasma with tumor cells was unlikely because

K-ras gene was detected in DNA only from patients with cancer and not from

Our finding, however, demonstrated the redetection of K-ras gene mutation in plasma DNA, in accordance with tumor relapse and recurrence. Therefore, both mechanisms could be responsible for the presence of cancer DNA in plasma or serum. Quantitative assay for mutated DNA may be helpful in understanding the mechanism.

MASA, followed by agarose gel electrophoresis, was used as the primary technique for detecting K-ras gene alterations in this study. This method is convenient and sensitive to detect micrometastasis (42, 43). MASA assay can detect as little as 0.01 ng of mutant DNA in 1 μg of normal DNA and routinely detected 0.1 ng of mutant DNA in 1 μg of normal DNA (25). Because MASA is extremely sensitive, it must be carefully optimized to minimize the possibility of false-positive reactions (44). However, it is unlikely that the results obtained in this study by MASA were false positive. Mutant K-ras gene was detected in DNA only from patients with cancer and not from our control subjects with chronic pancreatitis or healthy individuates. Furthermore, we confirmed that contamination of plasma with tumor cells was unlikely because K-ras mutations were not detected in DNA from mononuclear cells.

Although CA19-9 is one of the useful markers for clinical outcome of patients with pancreatic adenocarcinoma (36), ~10% of the population does not produce CA19-9 because of individual genetic alterations for Lewis enzyme expression (45). Serum CA19-9 levels often increase in patients with pancreatitis and obstructive jaundice, which are often associated with pancreatic cancer (46). We did not always observe the close association of serum CA19-9 level with positivity of K-ras gene mutation in plasma. In those patients, detection of K-ras mutation in plasma DNA could be a more useful marker than serum CA19-9 levels.

In conclusion, our study demonstrated that circulating mutated DNA of the K-ras gene is present in plasma of patients with pancreatic adenocarcinoma and that the appearance of K-ras gene mutation in plasma DNA may reflect the tumor size. The presence of mutant plasma DNA in patients with pancreatic adenocarcinoma, including those with potentially curative disease, suggests that this assay of plasma samples may be clinically helpful for the diagnosis, evaluation of tumor burden, and indication of surgical resection for these tumors. Further studies using a larger population sample are necessary to establish the clinical significance of mutant plasma DNA in patients with pancreatic adenocarcinoma.

ACKNOWLEDGMENTS

We thank all members of the Osaka University Hospital, Department of Surgery, Osaka National Hospital, and Moriguchi Keijin-ka Hospital for providing tissue samples and the staff of the Department of Diagnostic Reagent Development, Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan), for technical support. We would also like to thank Dr. F. G. Issa (University of Sydney, Sydney, Australia) for the careful reading and editing of this manuscript.

REFERENCES


Table 4 Summary of clinical and histopathological findings in 15 patients with K-ras mutations in pancreatic ductal adenocarcinoma

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Treatment</th>
<th>Tumor size (cm)</th>
<th>TNM classification</th>
<th>Histological type</th>
<th>K-ras gene mutation</th>
<th>Tumor marker (units/ml)</th>
<th>Clinical outcome</th>
<th>Recurrence site</th>
<th>Survival (months)</th>
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<tr>
<td>1</td>
<td>55</td>
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<td>1.5</td>
<td>T2 N0 M0</td>
<td>I</td>
<td>well</td>
<td>+</td>
<td>&lt;37 &lt;37</td>
<td>NED</td>
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<td>3.0</td>
<td>T1 N0 M0</td>
<td>II</td>
<td>well</td>
<td>+</td>
<td>&lt;37 &lt;37</td>
<td>NED</td>
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<tr>
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<td>M</td>
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<td>well</td>
<td>+</td>
<td>&lt;37 &lt;37</td>
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<tr>
<td>4</td>
<td>70</td>
<td>F</td>
<td>PD, curative</td>
<td>2.2</td>
<td>T1 N0 M0</td>
<td>III</td>
<td>well</td>
<td>+</td>
<td>&lt;37 &lt;37</td>
<td>DOD Local</td>
<td>19.3</td>
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<td>5</td>
<td>64</td>
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<td>DP, palliative</td>
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<td>1400 4200</td>
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<td>well</td>
<td>+</td>
<td>630 6100</td>
<td>DOD</td>
<td>1.3</td>
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a PD, pancreatoduodenectomy; DP, distal pancreatectomy; curative, curative resection; palliative, palliative resection; well, well-differentiated adenocarcinoma; moderate, moderately differentiated adenocarcinoma; NED, no evidence of disease; DOD, death due to pancreatic adenocarcinoma; AWD, alive with disease.

b K-ras gene mutation in plasma DNA pre- and posttreatment (1-2 months after). +, presence; –, absence.

c Normal range, <37 units/ml.
d Death due to other cause (sepsis).


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