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.
PATIENTS AND METHODS

Patients. A total of 30 subjects were recruited in this study, including 21 patients with pancreatic adenocarcinoma, 4 patients with chronic pancreatitis, and 5 age-matched healthy subjects. All patients were treated with pancreatectomy or laparotomy at Department of Surgery II, Osaka University Medical School, and affiliated hospitals between January 1, 1994, and April 30, 1997. 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 TNM 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 mean size of the tumor was 3.9 cm in diameter, ranging from 1.5 to 9.0 cm. The disease stage was classified to lymph nodes or distant organs. Sixteen tumors were located in 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, Upppsala, 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. Oligonucleotide sequences for MASA

<table>
<thead>
<tr>
<th>Type of primer</th>
<th>Sequences&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type primers</td>
<td>5'-ACT TGT GGT AGT TGG AGC TGG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CTC ATG AAA ATG GTC AGA GAA ACC-3'</td>
</tr>
<tr>
<td>MASA primers</td>
<td>5'-ACT TGT GGT AGT TGG AGC TCG-3'</td>
</tr>
<tr>
<td>Set 1, for detection of first-letter mutation (forward)</td>
<td>5'-ACT TGT GGT AGT TGG AGC TTT-3'</td>
</tr>
<tr>
<td>Set 2, for detection of second-letter mutation (forward)</td>
<td>5'-ACT TGT GGT AGT TGG AGC TTA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CTT GTG GAT TAA</td>
</tr>
<tr>
<td></td>
<td>5'-CTT GTG GAT TAA</td>
</tr>
<tr>
<td></td>
<td>5'-CTT GTG GAT TAA</td>
</tr>
</tbody>
</table>

<sup>a</sup>The underlined letters indicate codon 12 of the K-ras gene, whereas the italicized letters indicate mutant-specific variants of the first (set 1) or the second (set 2) nucleotide of codon 12 in the K-ras gene.

in patients with pancreatic adenocarcinoma and assessed its clinicopathological value in these patients.

Statistical Analysis of Survival Curves. Statistical comparisons of baseline data between groups were tested by $\chi^2$
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 restested 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...
cheto-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.

First, 9 of 15 (60%) patients with pancreatic adenocarcinoma who had K-ras gene mutations in primary tumor had identical mutations in plasma DNA. Second, these mutations were not detected in DNA from mononuclear cells which might be considered as normal cells, suggesting that the mutated DNA was not contaminated into normal cells at the time of sample collection. Third, mutations were not detected after tumor resection or intensive chemo- or radiotherapy in 6 of 9 patients who were positive for mutation in plasma DNA. Fourth, our correlation analysis suggested that detection of the K-ras mutation in plasma DNA identical to that present in the primary tumor should be clinically useful for determining the size of the tumor and predict surgical resectability and prognosis. Patients who were positive for K-ras gene mutations in plasma DNA 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.

Although the presence of cancer DNA in plasma or serum has been recently described (30–32, 40), the underlying process that causes free 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 may be favorable to this possibility. On the other hand, another possible mechanism raised is that live cancer cells actively secrete DNA. Leon et al. (37) reported that patients responding to radiotherapy usually showed decreasing plasma DNA levels, whereas increasing levels would be expected if tumor cell lysis were responsible for the phenomenon. In this study, K-ras gene mutation in plasma disappeared after chemo-radiotherapy.

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**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>
</tr>
<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.*

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**Fig. 2** Ethidium bromide staining of PCR products for each mutant-specific primer. MASA-PCR products for each mutant-specific primer were electrophoresed in 2% agarose gels containing 1 μg/ml ethidium bromide. Lane M, size marker (1-kb DNA ladder); Lane PC, positive control. Note that, in this case, the mutation in PCR product from plasma DNA is similar to that in the primary tumor (GGT to CGT).
Our finding, however, demonstrated the redetection of \textit{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 \textit{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 (27). 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 \textit{K-ras} gene was detected in DNA only from patients with cancer and not from our control subjects with chronic pancreatitis or healthy individuals. Furthermore, we confirmed that contamination of plasma with tumor cells was unlikely because \textit{K-ras} mutations were not detected in DNA from mononuclear cells.

Although \textit{CA19-9} is one of the useful markers for clinical outcome of patients with pancreatic adenocarcinoma (36), \textsim{}10% of the population does not produce \textit{CA19-9} because of individual genetic alterations for Lewis enzyme expression (45). Serum \textit{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 \textit{CA19-9} level with positivity of \textit{K-ras} gene mutation in plasma. In those patients, detection of \textit{K-ras} mutation in plasma DNA could be a more useful marker than serum \textit{CA19-9} levels.

In conclusion, our study demonstrated that circulating mutated DNA of the \textit{K-ras} gene is present in plasma of patients with pancreatic adenocarcinoma and that the appearance of \textit{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.

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