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Detection of Hepatic Micrometastasis in Pancreatic Adenocarcinoma Patients by Two-stage Polymerase Chain Reaction/Restriction Fragment Length Polymorphism Analysis

Soichiro Inoue, Akimasa Nakao, Yasushi Kasai,[1] Akio Harada, Toshiaki Nonami and Hiroshi Takagi

Department of Surgery II, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466

Hepatic metastasis and retroperitoneal recurrence generally are considered to be the two primary modes of recurrence in pancreatic cancer. The goal of this study was to determine if patients with pancreatic adenocarcinoma have hepatic and peritoneal micrometastasis at operation. Pancreatic adenocarcinomas are known to have a high incidence of K-*ras* gene mutations. Liver tissue specimens were obtained from 30 patients (17 with pancreatic adenocarcinoma and 13 with other diseases) with a biopsy needle at operation. Peritoneal washings were obtained during operation from 20 patients with pancreatic adenocarcinoma. Two-stage polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis were used to detect K-*ras* oncogene mutation at codon 12. Thirteen of 17 pancreatic adenocarcinoma patients had K-*ras* gene mutations in the liver, whereas all 13 patients with other diseases did not. However, only two of 20 pancreatic adenocarcinoma patients revealed K-*ras* gene mutation in peritoneal lavage fluids. These results indicate the feasibility of detecting hepatic micrometastasis in patients with pancreatic adenocarcinoma, and imply that PCR/RFLP analysis may be of value in the diagnosis, treatment and follow-up of hepatic metastasis of pancreatic adenocarcinoma.

Key words: Pancreatic cancer — K-*ras* — PCR/RFLP analysis — Micrometastasis

The prognosis of patients with pancreatic adenocarcinoma after operation remains poor in spite of the development of diagnostic imaging, surgical techniques and so on.[1)] The main cause of death is hepatic metastasis and retroperitoneal recurrence. Fortner has reported that recurrent disease almost always manifests as abdominal carcinomatosis, with liver metastases present in approximately half of such patients.[2)] This finding makes it difficult to determine whether many of the hepatic metastases had been initiated before resection, or whether tumor cells had migrated intraoperatively as a result of surgical manipulation. This has long been a key issue among surgeons. We hypothesized that even if hepatic metastases are not apparent before and during operation, there is still a possibility that hepatic micrometastasis is present in patients with pancreatic adenocarcinoma at operation.

The multiple polymerase chain reaction (PCR) procedures developed for the detection of point-mutated genes in the presence of an excess of unaltered genes have been reviewed.[3)] Over 90% of pancreatic adenocarcinomas contain mutated *ras* genes, and the site of mutation was restricted to codon 12 of the K-*ras* gene.[4–6)] Since mutant K-*ras* is not found in benign disease, such as chronic pancreatitis, the detection of mutant K-*ras* in pancreatic disease provides a definitive diagnosis of pancreatic adenocarcinoma.[6,7)] Recently, Kahn *et al.* developed a rapid and highly sensitive PCR/RFLP (restriction fragment length polymorphism) assay for the detection of a mutant codon 12 human K-*ras* allele.[8)] Hence, using two-stage PCR/RFLP analysis, we evaluated DNA samples extracted from liver tissue and peritoneal lavage fluid of patients to determine if micrometastatic cells containing a K-*ras* mutation could be detected.

Liver tissue specimens were obtained from thirty patients within two years using a 14 gauge biopsy needle soon after laparotomy at operation. We kept the tissue on ice and began DNA extraction as soon as possible. To study K-*ras* mutations in the main tumors, several 50-μm tissue sections were cut from each of the tissue blocks of resected pancreatic adenocarcinomas. Adjacent sections were stained with hematoxylin and eosin to confirm the presence of carcinoma tissue at this location.

Peritoneal washings were further collected from twenty patients with pancreatic adenocarcinoma within two years. In laparotomy patients, particular attention was paid to hemostasis during incision of the abdominal wall. Then, sufficient free fluid was obtained, and about 200 ml of isotonic heparinized saline was introduced into

[1] To whom correspondence should be addressed.

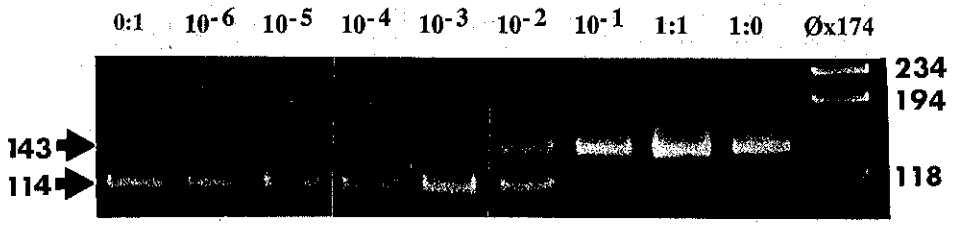

Fig. 1. Two-stage PCR/RFLP analysis of titration test. SW480 DNA was mixed with HT29 DNA in the following ratios: 1 μg/0; 0.5 μg/0.5 μg; 100 ng/1 μg; 10 ng/1 μg; 1 ng/1 μg; 100 pg/1 μg; 10 pg/1 μg; 1 pg/1 μg; 0/1 μg. Ratios of SW480 to HT29 DNA are given above the sample lanes. Øx174: Øx174 *Hae*IIIcut DNA marker.

the subphrenic space and Douglas cavity. After gently churning, as much fluid as possible was recovered using a syringe and quill and transported to the laboratory. Extraction of DNA from liver tissue material, paraffin-embedded tissue and peritoneal lavage fluid material was carried out as previously described.[9]

Extracted DNA from each case was evaluated by two-stage PCR amplification and RFLP analysis with nucleotide substitution in PCR primers, creating *Bst*NI (New England Biolabs., Beverly, MA) restriction patterns that distinguished mutated from normal alleles as previously described.[8] First-stage PCR amplification was performed on 100 ng of high-molecular-weight DNA. We always ran these procedures in triplicate, and the accuracy of the two-stage PCR/RFLP assay was validated with DNA from SW480 and HT29 colonic carcinoma cell lines, as positive and negative controls, with known mutated and wild-type K-*ras* gene sequences.[10]

The PCR products of the 143-base pair (bp) mutant band were subcloned by a TA cloning strategy (Invitrogen Inc., San Diego, CA) using the manufacturer's recommended protocol. At least five clones were sequenced by the double-stranded dideoxy method. The sequencing reactions were carried out on ABI Catalyst (Applied Biosystems, Foster City, CA) using fluorescently labeled universal and reverse primers, respectively. The sequences were collected on an ABI 373A sequencer (Applied Biosystems).

Sensitivity was tested with a series of titration experiments. To determine the pattern of amplified DNA fragments produced in a two-stage PCR/RFLP analysis, mixtures of DNA containing wild type K-*ras* codon 12 sequences (HT29) were made with serial dilutions of SW480 DNA. The mutant K-*ras* allele could be detected in mixtures incorporating as little as 100 pg of SW480 DNA, as indicated by the appearance of a 143-bp band. Two-stage PCR/RFLP analysis could detect even one K-*ras* mutated cell in a 10^4 cell population (Fig. 1).

In liver tissue specimens, mutations of K-*ras* codon 12 were detected in 13 of 17 cases (76.5%) with pancreatic adenocarcinoma (Fig. 2, Table I), but in none of the 13

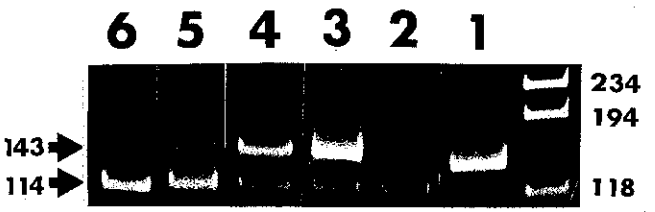

Fig. 2. Photograph of DNA electrophoregram on a 15% native PAGE gel after ethidium bromide staining and UV analysis. Right lane: molecular weight markers (*Hae*III-digested Øx174 DNA markers). Lane 1: *Bst*NI digestion of two-stage PCR product from homozygous mutated-type codon 12 K-*ras* gene showing a 143-bp fragment (SW480 cell line). Lane 2: *Bst*NI digestion of two-stage PCR product from wild-type codon 12 K-*ras* gene showing a 114-bp fragment (HT29). Lane 3: *Bst*NI/RFLP from two-stage PCR products after DNA amplifications from liver tissue of patients with pancreatic adenocarcinomas showing 114-bp and 143-bp fragments (strongly positive case). Lane 4: same as above (moderately positive case). Lane 5: same as above (weakly positive case). Lane 6: same as above (negative case).

control cases (2 cases with chronic pancreatitis, 2 cases with benign papillary neoplasm of the pancreatic duct, 2 cases with chronic hepatitis, 2 cases with liver cirrhosis, 1 case with hepatocellular carcinoma, 1 case with duodenal adenocarcinoma, 1 case with choledocholithiasis, 1 case with papilla Vater carcinoma and 1 case with gastric cancer with hepatic metastases).

Then, in order to identify the exact mutation of K-*ras* codon 12 in these cases, the amplified products eluted from the mutant band were analyzed by DNA subcloning and sequencing. All the mutations were single nucleotide substitutions; no multiple substitutions within a single clone were found (Table I). In 5 of the 13 cases, the mutation was from a normal codon for glycine (GGT) to aspartic acid (GAT). Four of the 13 mutated to valine (GTT), three mutated to serine (AGT) and one case (No. 2) contained double mutations; glycine (GGT) to aspartic acid (GAT) and serine (AGT) at codon 12 of K-*ras*.

Table I. Individual Results for Liver Tissues of Patients with Pancreatic Adenocarcinoma

Case No.	Age(yr) /Sex	TNM clinical classification[a)]	Stage grouping	Operation	Macroscopic hepatic metastasis	K-*ras* mutation in liver tissue	Tumor mutation	Survival interval (months)	Outcome
1	69/M	T3NXM1	IV	Resected	+	GTT	GTT	1	Dead
2	63/M	T3N1M0	III	Resected	−	AGT,GAT	AGT,GAT	22	Alive
3	49/M	T3N1M0	III	Resected	−	GAT	GAT	9	Alive
4	47/M	T1bN1M0	III	Resected	−	GAT	GTT	9	Dead
5	59/M	T3N1M0	III	Resected	−	AGT	GAT,GTT	7	Alive
6	50/M	T1aN1M0	III	Resected	−	AGT	AGT,GAT,GTT	5	Alive
7	52/M	T3N1M0	III	Resected	−	GTT	GTT	5	Alive
8	74/M	T3AnyNM0	III	Palliative	−	GAT	GAT	6	Dead
9	57/F	T3NXM0	−	Palliative	−	AGT	N/A[b)]	4	Alive
10	36/M	T3NXM0	IV	Palliative	−	GAT	GAT	5	Alive
11	78/M	T2NXM0	IV	Palliative	−	GTT	GTT	5	Alive
12	38/M	T3NXM1	IV	Palliative	+	GTT	GTT	7	Dead
13	68/M	T3NXM1	IV	Palliative	+	GAT	GAT	6	Alive
14	60/M	T3N1M0	III	Resected	−	−	GAT	18	Alive
15	77/M	T2N1M0	III	Resected	−	−	AGT,GTT	Operative	Dead
16	70/M	T2N1M0	III	Resected	−	−	GTT	3	Alive
17	63/F	T3NXM0	IV	Palliative	+	−	N/D[b)]	5	Dead

a) According to the 1987 UICC TNM classification of malignant tumors.
b) N/A, not available; N/D, not done.

Table II. Individual Results for Peritoneal Lavage of Patients with Pancreatic Adenocarcinoma

Case No.	Age(yr) /Sex	TNM clinical classification[a)]	Stage grouping	Operation	Macroscopic peritoneal dissemination	K-*ras* mutation in peritoneal lavage
1	69/M	T3NXM1	IV	Resected	+	GTT
2	79/M	T3NXM1	IV	Palliative	+	CGT,AGT
3	63/M	T3N1M0	III	Resected	−	−
4	49/M	T3N1M0	III	Resected	−	−
5	47/M	T1bN1M0	III	Resected	−	−
6	59/M	T3N1M0	III	Resected	−	−
7	50/M	T1aN1M0	III	Resected	−	−
8	52/M	T3N1M0	III	Resected	−	−
9	60/M	T3N1M0	III	Resected	−	−
10	77/M	T2N1M0	III	Resected	−	−
11	70/M	T2N1M0	III	Resected	−	−
12	63/M	T4N2M0	IV	Resected	−	−
13	76/F	T3N1M0	III	Resected	−	−
14	74/M	T3AnyNM0	III	Palliative	−	−
15	57/F	T3NXM0	−	Palliative	−	−
16	36/M	T3NXM0	IV	Palliative	−	−
17	78/M	T2NXM0	IV	Palliative	−	−
18	38/M	T3NXM1	IV	Palliative	−	−
19	68/M	T3NXM1	IV	Palliative	−	−
20	63/F	T3NXM1	IV	Palliative	−	−

a) According to the 1987 UICC TNM classification of malignant tumors.

Genomic DNA was obtained from 15 pancreatic tumors, and the spectrum of K-*ras* mutations observed in our analysis is summarized in Table I. Six of the 15 had mutated to valine (GTT), five to aspartic acid (GAT) and four of the tumors contained more than one mutation. We compared the type of mutations in liver tissue samples and the main tumors in 12 cases. In ten of the 12 cases (Nos. 1, 2, 3, 6, 7, 8, 10, 11, 12 and 13), the type of

mutation of liver samples was the same as that of the main tumors. However, in two cases (Nos. 4 and 5) the mutations of liver samples were different from those of main tumors.

On the contrary, from peritoneal lavage fluid materials, mutations of K-*ras* codon 12 were detected in only two cases (10%) with pancreatic adenocarcinoma. One mutated to valine (GTT) and the other mutated to arginine (CGT) (Table II). Overall, four different types of base changes were found, causing a substitution of wild-type glycine (GGT) to either aspartic acid (GAT), valine (GTT), serine (AGT) or arginine (CGT).

The advent of genomic DNA amplification by PCR has been extremely valuable in many areas of molecular biology. The presence of K-*ras* mutations in specimens obtained by needle aspiration and biopsy has been studied as an aid to the pathological diagnosis of pancreatic cancer.[6, 11, 12] More recently, K-*ras* mutations have been detected in pancreatic secretions from patients with pancreatic adenocarcinoma and pancreatic intraductal neoplasia.[13, 14] Therefore, a simple and sensitive assay could become an important clinical tool in the detection and monitoring of cells harboring activated K-*ras* genes at early stages of the transformation process. For example, using a mutant allele-specific amplification method, Hayashi *et al.* recently detected mutant K-*ras* and p53 genes from colorectal cancers in lymph node.[15]

Kahn *et al.* recently developed a rapid and highly sensitive nonradioactive method for the detection of a mutant codon 12 human K-*ras* allele in the presence of as many as 10^4 copies of the wild-type codon 12 allele.[8] This sensitivity was achieved by selective PCR amplification of mutant K-*ras* gene sequences employing a two-stage procedure. Using this two-stage PCR/RFLP analysis, we detected micrometastatic cells in liver tissue of 13 of 17 patients with pancreatic adenocarcinoma at the time of operation. This easy and sensitive assay could prove to be valuable in the clinical detection of pre-malignant lesions in organs known to give rise to tumors that contain activated K-*ras* genes. In addition, this PCR/RFLP analysis demonstrated that in patients without macroscopic hepatic metastasis, a small number of pancreatic cancer cells had already spread to the liver tissue. We hypothesize that these cancerous cells are trapped at the liver sinusoids, though no pathological evidence is yet available. In fact, we could detect a K-*ras* oncogene mutated at codon 12 in DNA purified from portal vein blood in Case No. 7 (Table I). These findings may be helpful for understanding possible patterns of hepatic metastasis of pancreatic cancer as well as for treating pancreatic cancer.

From our intraoperative biopsy of the site of macroscopic hepatic metastasis, this site proved to be adenocarcinoma histologically in Case No. 17 (Table I). Genomic DNA extracted from this site contained K-*ras* codon 12 mutations. The reason for this contradictory result may be a sampling problem: some needle biopsy specimens may not contain any cancerous cells or the sensitivity of this two-stage PCR/RFLP analysis may be insufficient in some cases.

The frequency pattern of mutations at codon 12 was somewhat different from those mentioned in previous reports on the similar analysis of pancreatic cancers in European countries[4, 16] and Japan.[17, 18] To our knowledge, a mutation consisting of a G-to-A transition in the first base of codon 12 of K-*ras*, which replaces a wild-type glycine with serine, has not yet been reported among human pancreatic cancers. However, this mutation of codon 12 of K-*ras* has been reported in various human cancers,[19–22] indicating that a putative glycine-to-aspartic acid or glycine-to-valine change is not necessarily the critical event for K-*ras* gene activation at codon 12. It has also been reported that several distinct K-*ras* mutations were simultaneously present in a single specimen of the tumor.[18, 19, 22] Whether these reports and our cases reflect the presence of multifocal carcinomas or, perhaps more likely, an invasion of K-*ras* mutation-positive duct lesions by a carcinoma having a different *ras* mutation, is difficult to ascertain.

We found discrepancies between the liver and the main tumor with respect to the type of K-*ras* mutation in Case Nos. 4 and 5 (Table I). However, the reason why the types of mutations of liver samples in these cases were different from those of the main tumors remains unknown. Using a plaque hybridization assay, Caldas *et al.* analyzed stool specimens for mutated K-*ras* sequences in pancreatic adenocarcinoma patients,[23] and compared the type of mutations between stool samples and the main tumors in six pancreatic cancer patients. In one case, a different type of K-*ras* mutation was found.

The encouraging results of the present study on only a small number of liver tissues analyzed should now be verified in a much larger number of pancreatic adenocarcinoma cases. Although it was reported that there was no statistically significant difference in the rate or positivity of mutations among the stages of the disease,[24] follow-up of the patients is most important in the present study. Case No. 4 (Table I), who underwent surgical resection, died of peritonitis carcinomatosa. At autopsy, multiple hepatic metastases were recognized. Nevertheless, the present investigation provides data, albeit preliminary, suggesting that two-stage PCR/RFLP analysis may be available as a clinical tool to offer more accurate assessment of hepatic metastasis of pancreatic cancer and a better biological understanding of the hematogenous dissemination of pancreatic adenocarcinoma.

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