RAPID COMMUNICATION Jpn. J. Cancer Res. 87, 793–797, August 1996

Detection of *ras* Gene Mutations in Perioperative Peripheral Blood with Pancreatic Adenocarcinoma

Shuji Nomoto, Akimasa Nakao, Yasushi Kasai, Akio Harada, Toshiaki Nonami and Hiroshi Takagi

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

Surgeons wish to know of any correlation between an operation and the incidence of metastasis. In perioperative periods, pancreatic cancer cells were identified by detecting mutant K-*ras* gene by two-step PCR and RFLP analysis in blood samples taken from peripheral blood. In no case was K-*ras* point mutation detected in blood before operation, although the mutant band was observed in all cases at the time the lesion was resected. Surprisingly, in five of ten cases, positive bands were identified just after laparotomy, before we had reached the primary lesion. In almost all cases, mutant K-*ras* was detected until the fourteenth postoperative day. These findings suggest that cancer cells exist in the circulation, and have a potential for hematogenous metastasis during the perioperative period. In conclusion, surgical stress causes hematogenous dissemination of pancreatic cancer cells, and surgeons should employ the appropriate anti-metastasis therapy in the perioperative period.

Key words: Pancreatic cancer — Blood — Metastasis — K-*ras* — PCR

Despite radical operation using the non-touch isolation technique,[1] pancreatic cancer frequently recurs in liver and peritoneum, and the prognosis remains very poor.[2] In some cases, one has the impression that the operation itself accelerates the recurrence. We suspect that the operation itself triggers the hematogenous spread of cancer cells, but little is known about the nature of this process. Detailed study of the appearance and biology of this important process has been limited by the methods available. Thanks to recent advances in *ras* gene studies, cancer cells can be detected in the peripheral blood by amplification of specific DNA sequences.[3] Since one would not expect epithelial cells normally to be present in the blood, genes specific to pancreatic cancer might be suitable targets to identify malignant circulating cells. Thus, we designed this prospective study to investigate the possible existence of K-*ras* point mutation in peripheral blood during the perioperative period, because we consider that detection of carcinoma cells in the circulation would demonstrate the need for appropriate therapy for prevention of parenchymatous metastases to the liver and other organs.

Peripheral blood was collected from ten Japanese pancreatic cancer patients at Nagoya University Hospital between August 1994 and July 1995. Blood was obtained before and during operation (A, just after laparotomy; B, upon resecting the primary lesion; C, at closure of abdominal wall), and at 1, 3, 7, 14 and 21 POD. As a control, we collected peripheral blood as described above from two patients with other diseases. One had chronic pancreatitis, and the other had undergone laparotomical biopsy of the pancreas for suspected pancreatic cancer, but no malignant lesion was detected. For reference, peripheral blood was also obtained in the same way from another patient with pancreatic cancer whose lesion had not revealed K-*ras* point mutation. Peripheral blood was drawn with a standard venipuncture technique. The drawn blood was immediately mixed with DTAB solution as described later. To study K-*ras* point mutation of primary tumor, the resected pancreatic adenocarcinoma was cut into small pieces and DNA was extracted.

Genomic DNA extraction from approximately 1 ml of whole blood was performed as described previously with a minor modification.[4] Two-stage PCR amplifications and RFLP analysis were carried out as previously described.[5] We always conducted these procedures in triplicate, and the accuracy of the two-step 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. PCR products were electrophoresed through a 15% native polyacrylamide gel. Gels were stained with ethidium bromide, and photographed on an ultraviolet light transilluminator.

To look for corresponding genetic alterations where point mutations in blood had been identified in primary tumors, after the second digestion, the products were

The abbreviations used are: PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; RFLP, restriction fragment length polymorphism; DTAB, dodecyltrimethylammonium bromide; POD, postoperative day; ICAM-1, intercellular adhesion molecule-1

electrophoresed and the 143-nt band was cut out and incubated with twice the volume of elution buffer (0.5 *M* ammonium acetate, 10 m*M* magnesium acetate, 1 m*M* EDTA [pH 8.0], 0.1% SDS) at 37°C for 3 h. The supernatant was precipitated with 2 volumes of 100% ethanol and centrifuged at 10000 rpm for 15 min. The pellet was rinsed with 70% ethanol, dried and resuspended in TE buffer. The recovered 143-base pair bands were subcloned by a TA cloning strategy (Invitrogen Inc., San Diego, CA) using the manufacturer's recommended protocol. At least five clones of each band 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. The sequences were collected on an ABI 373A sequencer (Applied Biosystems).

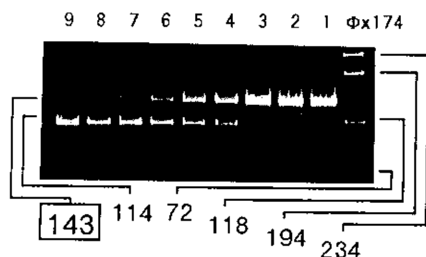

Fig. 1. Sensitivity of the two-stage PCR/RFLP analysis. SW480 DNA was mixed with HT29 DNA in the following ratios: Right lane: molecular weight markers (*Hae* III-digested Φx 174 DNA markers). Lane 1: 1μg/0 (1:0). Lane 2: 0.5 μg/0.5 μg (1:1). Lane 3: 250ng/1μg (1/4). Lane 4: 100 ng/1 μg (10^{-1}). Lane 5: 10 ng/1 μg (10^{-2}). Lane 6: 1 ng/1 μg (10^{-3}). Lane 7: 100 pg/1 μg (10^{-4}). Lane 8: 10 pg/1 μg (10^{-5}). Lane 9: 0/1 μg (0:1). This method could detect 10^{-4} mutated cells in the wild-type cells.

Sensitivity was tested by means of 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 DNA extracted from SW480. 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 the 143-bp band. Two-stage PCR/RFLP analysis could detect a single K-*ras* mutated cell in a 10^4 cell population (Fig. 1). In this study, the positive band was identified in all cases just when resecting the primary lesion. However, in five of ten cases, mutant K-*ras* was detected immediately after the laparotomy (Fig. 2).

The band weakened day by day, but was still visible by POD fourteen (Fig. 3). On the twenty-first day, a positive band could no longer be identified in nine of ten cases.

In the control group, we could not detect the mutant band at any time in the perioperative period. Similarly,

Fig. 2. Photograph of DNA electrophoretogram on 15% native PAGE gel after ethidium bromide staining and UV analysis. Detection of K-*ras* point mutation in peripheral blood just after laparotomy in ten Japanese pancreatic adenocarcinoma patients. Right lane: molecular weight markers (*Hae* III-digested Φx 174 DNA markers). Lane 1: case No. 1 (positive). Lane 2: case No. 2 (positive). Lane 3: case No. 3 (positive). Lane 4: case No. 4 (negative). Lane 5: case No. 5 (positive). Lane 6: case No. 6 (negative). Lane 7: case No. 7 (negative). Lane 8: case No. 8 (negative). Lane 9: case No. 9 (positive). Lane 10: case No. 10 (negative). Lane 11: BstNI digestion of two-stage PCR product from homozygous mutated-type codon 12 K-*ras* gene showing a 143-bp positive fragment (SW480 cell line).

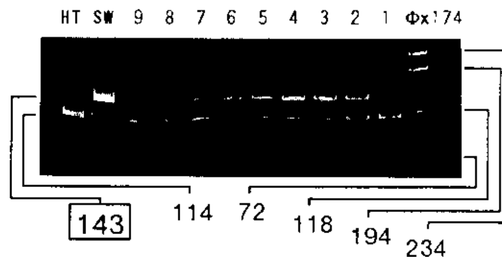

Fig. 3. Photograph of DNA electrophoretogram on a 15% native PAGE gel after ethidium bromide staining and UV analysis. Detection of K-*ras* point mutation in peripheral blood of perioperative period in case No. 1. Right lane: molecular weight markers (*Hae* III-digested Φx 174 DNA markers). Lane 1: before operation (negative). Lane 2: just after laparotomy (positive). Lane 3: just upon resecting the primary lesion (positive). Lane 4: at closure of abdominal wall (positive). Lane 5: POD one (positive). Lane 6: POD 3 (positive). Lane 7: POD 7 (positive). Lane 8: POD 14 (weakly positive). Lane 9: POD 21 (negative). Lane 10: BstNI digestion of two-stage PCR product from homozygous mutated-type codon 12 K-*ras* gene showing a 143-bp fragment (SW480 cell line). Lane 11: BstNI digestion of two-stage PCR products from wild-type codon 12 K-*ras* showing a 114-bp fragment (HT29). The 143-bp band was detected from just after laparotomy to POD 14.

Table I. K-*ras* Point Mutation Results in Perioperative Peripheral Blood

Case No.	Age Sex	TNM clinical classification	Stage grouping	Operation	Preoperative	Intraoperative[a]			POD					Blood mutation	Tumor mutation	Survival interval	Outcome
						A	B	C	1	3	7	14	21				
1	67F	T3N0M0	II	Resected	−	+	+	+	+	+	+	±	−	GTT	GTT	10 mo	dead
2	68M	T1N0M0	I	Resected	−	+	+	+	+	+	+	+	−	AGT	AGT	19 mo	alive
3	69M	T3N0M0	II	Resected	−	+	+	+	+	+	+	+	−	GAT	GAT, AGT	14 mo	dead
4	47M	T1N1M0	III	Resected	−	−	+	+	+	+	+	+	−	GTT	GTT	16 mo	dead
5	59M	T3N1M0	III	Resected	−	+	+	±	±	±	+	±	−	GAT, AGT	GAT, AGT	3 mo	dead
6	75M	T2N1M0	III	Resected	−	−	+	+	+	+	+	+	−	CGT	CGT	15 mo	alive
7	63F	T3N1M1	IV	Resected	−	−	+	+	+	+	+	+	−	GAT	GAT	15 mo	alive
8	64F	T3N1M0	III	Resected	−	−	+	+	+	+	+	+	+	GAT, AGT	GAT, AGT	13 mo	alive
9	54M	T3N0M0	II	Resected	−	+	+	+	+	+	+	+	−	GTT	GTT	12 mo	alive
10	69M	T3N0M0	II	Resected	−	−	+	+	+	+	+	+	−	GTT	GTT	6 mo	dead

According to 1987 UICC TNM Classification of Malignant Tumors.
a) A, just after laparotomy; B, upon resecting the primary lesion; C, at closure of abdominal wall.

the mutant K-*ras* band was never identified in the pancreatic cancer case without K-*ras* point mutation.

Finally, four different base changes were detected, resulting from substitution of wild-type glycine (GGT) for aspartic acid (GAT), valine (GTT), serine (AGT) or arginine (CGT). In all cases, the results for the mutant sequence from collected blood accorded with those for the primary tumor. Table I summarizes the data.

Surgeons especially wish to know of any correlation between operation and the incidence of metastasis. Poste and Fidler reported that the process of metastasis is composed of sequential steps as follows: 1) malignant cells are released from the primary tumor and invade the surrounding host tissue; 2) invading tumor cells enter the lymphatics or blood vessels and are transported to distant sites in the body; 3) they lodge, arrest and adhere to the capillary bed of various organs; and 4) they exit from capillaries into the surrounding host tissue.[6] Many studies have been done on metastasis. Glaves *et al.* counted cancer cells in blood samples taken from the renal vein using a density gradient centrifugation procedure, and confirmed their identity using immunocytochemical techniques on the basis of their cytoskeletal intermediate filament proteins.[7] Cancer cells were found to be released as single cells and multicell emboli in 80% of patients who underwent radical nephrectomy for primary renal cancer. The results of their study confirmed that the cancer cells flow into the blood circulation under mechanical stress, such as an operation. But the method used was exceedingly complicated and thus impractical.

Recent studies have shown that the K-*ras* oncogene is activated by specific point mutations involving codon 12 in the large majority (up to 95%) of adenocarcinomas of the pancreas.[8–11] So it can be utilized directly only when cancer-specific abnormalities in DNA have been characterized. Since we would not expect epithelial cells to be normally present in the peripheral blood, genes specific to these tissues might be suitable targets for the detection of malignant circulating cells. PCR could enable detection of cancer cells in the blood by amplification of specific DNA sequences.[12]

Recently Moreno *et al.* reported the ability to detect hematogenous micrometastasis in prostate carcinoma.[13] Their data suggested that this metastasis may not be a terminal event in the natural history of human prostate cancer. Moreover, the results have important implications for the understanding and treatment of various cancers. The finding of cancer cells in the peripheral blood suggests that these cells circulate through all organ capillary beds during the metastatic process.

One of the initial steps in the metastatic process, of course, is the release of cancer cells from the primary tumor, but there have been few systematic investigations into quantitation of cancer cells. There has been speculation that mechanical stress promotes release of cancer cells. Yet, in the present study, circulating cancer cells were observed even before the surgical procedures had reached the primary tumor. Stress, such as anesthesia or laparotomy, apparently causes shedding of the cancer cells into the circulation.

In almost all cases of this study, cells of the primary cancer lesion invaded lymphatic or blood vessels. It appeared that preoperative stresses, such as ERP (endoscopic retrogradal pancreatography), serve to accelerate the release of cancer cells from the primary lesion into the blood circulation. The majority of these cancer cells in the circulation presumably flow to the first capillary bed, in liver, and are trapped there. Inoue *et al.* reported detection of DNA which has mutated K-*ras* in liver tissue.[11] But we consider the cancer cells in the

vasculature, such as the primary lesion or liver capillaries, to exist in conditions different from those in the circulation in general. Eventually, there are large numbers of cancer cells in the vasculature, but not in the circulation. In the circulation, supposing that the volume of whole blood is ten liters, we could detect mutated K-*ras* of 10^7 cancer cells among the wild-type K-*ras* of nuclear cells, such as white blood cells, based on the sensitivity. But there might well be fewer cancer cells than this in the circulation. This is presumably why the mutant band was not detected preoperatively. We speculate that the mutant K-*ras* DNA in the circulation arose from: 1) cancer cells released from the primary lesion, 2) cancer cells released from the capillary bed preoperatively (e.g., in liver or lymph nodes), and 3) DNA which had been in cancer cells scavenged by macrophages.

We must also consider two possible effects of anesthesia. The first is dynamic changes in the general and local circulation, such as in the primary cancer lesion and liver, where cancer cells presumably existed preoperatively. Secondly, some reports have indicated that anesthetic drugs affect ICAM-1 levels and postoperative metastases.[14, 15]

Our study showed that in almost every case, mutant K-*ras* could be detected up to fourteen days after operation. Studies with experimental animal tumors indicate that 70–95% of cancer cells given by tail vein injection are arrested in the first downstream capillary bed, the pulmonary microvasculature. The vast majority of the cancer cells arrested in the lungs are rapidly killed due to a variety of mechanical and host-mediated factors.[16] Certainly, there is a possibility that we detected mutant K-*ras* in macrophages after the cancer cells had been engulfed. But, the condition of the experimental animals in such studies would be different from that after a radical operation. We speculate that the circulating cancer cells may be trapped somewhere in the capillary bed, not killed, and later they may be re-released into the circulation. Following intramuscular injection of melanoma cells into mice, Glaves found circulating cancer cells, recovered from blood samples taken from the right ventricle, from day 3 through day 35.[17]

In conclusion, we have demonstrated that cancer cells flow into the circulation from the early stages of the operation upon laparotomy. There is a possibility that laparotomy promotes hematogenous dissemination of pancreatic cancer cells. DNA with K-*ras* point mutation exists in the circulation for at least fourteen POD, and may have the potential for causing metastasis. Thus, we recommend that adjuvant therapy, such as anticancer or anti-metastasis chemotherapy, be combined with radical operation from an early stage in the perioperative period.

(Received March 18, 1996/Accepted May 20, 1996)

REFERENCES

1) Nakao, A. and Takagi, H. Isolated pancreatectomy for pancreatic head carcinoma using catheter bypass of the portal vein. *Hepato-Gastroenterology,* **40,** 426–429 (1993).
2) Nakao, A., Harada, A., Nonami, T., Kaneko, T., Inoue, S. and Takagi, H. Clinical significance of portal invasion by pancreatic head carcinoma. *Surgery,* **117,** 50–55 (1995).
3) Tada, M., Omata, M., Kawai, S., Saisho, H., Ohto, M., Saiki, K. and Sninsky, J. J. Detection of *ras* gene mutations in pancreatic juice and peripheral blood of patients with pancreatic adenocarcinoma. *Cancer Res.,* **53,** 2472–2474 (1993).
4) Gustincich, S., Manfioletti, G., Del Sal, G., Schneider, C. and Carninci, P. A first method for high-quality genomic DNA extraction from whole human blood. *BioTechniques,* **11,** 298–302 (1991).
5) Kahn, S. M., Jiang, W., Culbertson, T. A., Weinstein, I. B., Williams, G. M., Tomita, N. and Ronai, Z. Rapid and sensitive nonradioactive detection of mutant K-*ras* genes via 'enriched' PCR amplification. *Oncogene,* **6,** 1079–1083 (1991).
6) Poste, G. and Fidler, I. J. The pathogenesis of cancer metastasis. *Nature,* **28,** 139–146 (1980).
7) Glaves, D., Huben, R. P. and Weiss, L. Haematogenous dissemination of cells from human renal adenocarcinomas. *Br. J. Cancer,* **57,** 32–35 (1988).
8) Almoguera, C., Shibata, D., Forrester, K., Martin, J., Arnheim, N. and Perucho, M. Most human carcinomas of the exocrine pancreas contain mutant c-K-*ras* genes. *Cell,* **53,** 549–554 (1988).
9) Yanagisawa, A., Ohtake, K., Ohashi, K., Hori, M., Kitagawa, T., Sugano, H. and Kato, Y. Frequent c-Ki-*ras* oncogene activation in mucous cell hyperplasias of pancreas suffering from chronic inflammation. *Cancer Res.,* **53,** 953–956 (1993).
10) Caldas, C., Hahn, S. A., Hruban, R. H., Redston, M. S., Yeo, C. J. and Kern, S. E. Detection of K-*ras* mutation and pancreatic ductal hyperplasia. *Cancer Res.,* **54,** 3568–3573 (1994).
11) Inoue, S., Nakao, A., Kasai, Y., Harada, A., Nonami, T. and Takagi, H. Detection of hepatic micrometastasis in pancreatic adenocarcinoma patients by two-stage polymerase chain reaction / restriction fragment length polymorphism analysis. *Jpn. J. Cancer Res.,* **86,** 626–630 (1995).
12) Smith, B., Selby, P., Southgate, J., Pittman, K., Bradley, C. and Blair, G. E. Detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction. *Lancet,* **338,** 1227–1229

(1991).
13) Moreno, J. G., Croce, C. M., Fischer, R., Monne, M., Vihko, P., Mulholland, S. G. and Gomella, L. G. Detection of hematogenous micrometastasis in patients with prostate cancer. *Cancer Res.*, **52**, 6110–6112 (1992).
14) Shapiro, J., Jersky, J., Katzav, S., Feldman, M. and Segal, S. Anesthetic drugs accelerate the progression of postoperative metastases of mouse tumors. *J. Clin. Invest.*, **68**, 678–685 (1981).
15) Azuma, K., Mike, N., Fujiwara, Y., Shimada Y. and Watanabe, T. Effect of halothane on intracellular adhesion molecule-1(ICAM-1) in melanoma cells. *J. Anesth.*, **7**, 442–446 (1993).
16) Fidler, I. J. Metastasis: quantitative analysis of distribution and fate of tumor emboli labeled with ^{125}I-5-iodo-2′-deoxyuridine. *J. Natl. Cancer Inst.*, **45**, 773–782 (1970).
17) Glaves, D. Metastasis: reticuloendothelial system and organ retention of disseminated malignant cells. *Int. J. Cancer*, **26**, 115–122 (1980).