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Detection of K-ras Codon 12 Mutation in the Portal Blood during Surgery for Pancreatic Carcinoma

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Abstract Background and Aim : It is well known that pancreatic adenocarcinomas harbor K-ras gene point mutations, which are frequently restricted to codon 12. Thus, the detection of K-ras codon 12 mutations in pancreatic or duodenal juice may aid in the diagnosis of pancreatic adenocarcinomas. In the present study, we investigated whether or not portal blood collected during surgery for pancreatic adenocarcinoma contains the K-ras codon 12 mutation.
Methods : During radical resection for pancreatic adenocarcinoma, 10ml of portal blood was taken twice ; first immediately after laparotomy, then just before resection. K-ras mutation was analyzed by a polymerase chain reaction using the primers corresponding to mutant alleles.
Results : K-ras mutation was identified in three of nine patients with pancreatic adenocarcinoma immediately before resection, while none of the patients were positive at the time of laparotomy. The K-ras status in the portal blood did not correlate with microscopic venous or lymphatic invasion.
Conclusion : K-ras mutations were identified in portal blood in some patients with pancreatic adenocarcinoma, which indicates that pancreatic cancer cells migrated into portal blood during resection and that some adjuvant therapy during surgery may be necessary to cure the pancreatic cancer.

Key Words: K-ras codon 12 mutation, liver metastasis, pancreas carcinoma, portal blood

Introduction

Invasive ductal adenocarcinoma of the pancreas is associated with a poor prognosis as most cases are unresectable by the time a diagnosis has been made using conventional methods, and surgical resection offers the only hope for long-term survival. In 1973, Fortner[1] first described performing regional pancreatectomy for cancer of the pancreas, since when the resection rate has been increasing. In addition to the improved safety of pancreatic resection, the improved survival of patients with pancreatic adenocarcinoma has been reported ; however, the 5-year survival rate remains as low as 15% to 27%[2,3], despite extensive surgery including resection of the retroperitoneal lymph nodes and nerve plexus around the celiac axis and superior mesenteric artery. The high incidence of recurrence in the liver after surgery is one dilenma preventing successful surgical treatment of pancreatic carcinoma. Although it is often suspected that occult liver metas-

tasis exists even before surgery, the exact mechanism of liver metastasis after surgery is unknown.

The K-ras gene encodes a 21-kilodalton guanosine triphosphatase protein, which regulates cell proliferation and differentiation via the tyrosine kinase-mediated signaling pathway[4]. Mutations of the K-ras gene result in excessive cell growth due to the decreased ability of mutant ras protein to interact with the guanosine triphosphatase activating protein. K-ras gene mutations are seen in about 50% of colorectal cancers, and about 30% of adenocarcinomas of the lung. The prevalence of K-ras gene mutation is more frequent. In fact, up to 90% of pancreatic adenocarcinomas have been shown to contain K-ras mutations[5,6]. Although mutations affecting codons 13 and 61 have been seen in a small subset of cases, most mutations are restricted to codon 12. Advances in molecular biological techniques have facilitated an increase in sensitivity, and these genetic characteristics have led to the detection of K-ras codon 12 mutation (K-ras mutation) in pancreatic juice[7] and duodenal juice[8] being used for the diagnosis of this carcinoma.

In the present study, we determined whether or not K-ras mutations were present in portal blood collected during surgery for pancreatic carcinoma as an genetic marker of pancreatic cancer cells to investigate the mechanism of liver metastasis and in order to know the influences of surgery we collected blood samples before and after the surgery.

Materials and Methods

The patients examined in the present study consisted of four men and five women ranging in age for 55 years to 70 years, with an average age of 64.7 years. Invasive ductal adenocarcinoma of the pancreas was pathologically proven in every patient, and radical resection was performed. The tumors were located in the head of pancreas in seven patients and in the body or tail in two. Every patient had associated regional lymph nodes metastasis. Pancreatoduodenectomy was performed in seven patients, preserving the pylorus in five, and pancreatectomy with splenectomy was performed in two patients. Portal veins with macroscopic tumorous involvement were resected and consequently reconstructed in three patients; two who underwent pancreatoduodenectomy and one who underwent distal pancreatectomy.

Twice during surgery, 10ml of whole blood was taken in the presence of ethylenediamine tetraacetic acid from the portal vein ; first immediately after laparotomy, then just before resection, and stored in a refrigerator at −80℃ until the analysis. The samples were centrifuged, and the supernatants discarded. The pellets were resuspended in 8ml of cold distilled water to lyse the red blood cells at 4℃ . After centrifugation and washing with phosphate-buffered saline, DNA was obtained by proteinase K digestion followed by phenol- chloroform extraction[9]. Polymerase chain reaction (PCR) was performed by the mutant-allele-specific amplification method[10]. The primers which correspond to the sequences of K-ras point mutation of codon 12 in the exon 1 were employed. The sequences of the primers were : 5' ACTTGTG GTAGTTGGAGCTC 3', 5' ACTTGTGGTA GTTGGAGCTT 3', 5' ACTTGTGGTAG TTGGAGCTA 3', and 5' CTTGTGGTA GTTGGAGCTGC 3', 5' CTTGTGGTAGTT GGAGCTGT 3', 5' CTTGTGGTAGTTGGA GCTGA 3' in set 1 and set 2, respectively ; and antisense primer : 5' GGTTTCTCTGACCAT TTTCATGAG 3'. For amplification of the wild-type K-ras gene, 5' AACTTGTGGTA GTTGGAGCTG 3' was used as a sense primer.

Amplification of an 179-, 178- or 180-base pair product from exon 1 was made by the primers. The PCR reaction mixture contained 100 ng of genomic DNA extracted from portal blood, 5μl of 10×reaction buffer (500mM KCl, 100mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$), 4μl of 2.5mM of each dNTP (dATP, dCTP, dTTP, dGTP), 2.0μM of each primer, 1.25units of Taq polymerase, and distilled water to give a final volume of 50μl . High-quality mineral oil (Sigma, St. Louis, MO) was layered on the top of the reaction mixtures to prevent evaporation. The PCR amplification was performed in a thermal cycle with 32 cycles at 95℃ for 30 seconds, at 54℃ , 64℃ , or 65℃ for 2 minutes in wild-type, set-1, or set-2 primer, respec-

tively, then at 70℃ for 2 minutes, using a RoboCycler (Stratagene Cloning Systems). Aliquots of 10 μl of the reaction mixtures were electrophoresed in 3% acrylamide gels and stained with ethidium bromide. K-ras mutation was determined to be positive when a specific band in the set-1 or set-2 primer PCR was seen in the gel.

The Fisher's exact probability test was used for evaluation and p-value less than 0.05 was regarded as statistically significant.

Results

Wild type K-ras gene was detected in every DNA sample (Fig. 1 panel A) and K-ras mutations were identified in three of the nine patients (positive cases). Case 4 and 8 had the point mutation in the 1st letter shown in the panel B and case 7 in the 2nd letter of K-ras codon 12 in the panel C. All of positive cases had tumors in the head of the pancreas (Table 1). Microscopically[11], no or minimal venous invasion and lymphatic permeation (v0,1 or ly0,1) was seen in two of positive cases (case 4 and 7). One of four patients with moderate to massive venous invasion or lymphatic permeation (v2,3 or ly2,3) had K-ras mutation (case 8). There was no statistically significant association between cancer invasion to the veins or lymphatic vessels and K-ras mutation positivity. From viewpoint of timing, all of the positive K-ras mutation were found at the time of resection, whereas

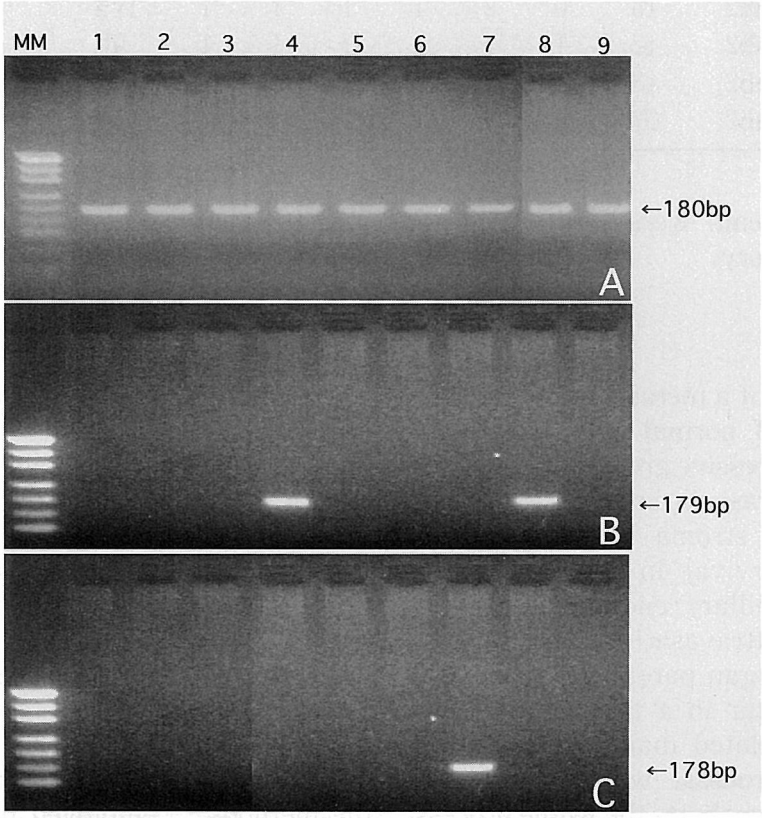

Fig.1 shows the photographs of K-ras codon 12 mutations in the portal blood determined by the mutation-allele-specific amplification method. The wild type of K-ras gene was detected in every sample as shown in the panel A. In the panel B and C, primers which correspond to the point mutation in the 1st and 2nd letter of codon 12, respectively, were employed for the polymerase chain reaction. K-ras mutation was positive when a specific band in the set-1 or set-2 primer PCR was seen in the gel as shown in the panel B and C. The first left lane in each panel represents the molecular bands. Positive cases in the panel B (case 4 and 8) and C (case 7) had been determined to have the point mutation in the 1st letter and the 2nd letter of K-ras codon 12, respectively. Lane numbers indicates the case numbers. MM, molecular marker

Table 1. demonstrates the clinicopathological feature based on the classification of pancreatic carcinoma according to the Japan Pancreas Society and the status of K-ras mutation. K-ras mutations were identified in the portal blood from 33% of the patients with pancreatic carcinoma, taken during radical resection. The letter of s, rp, n, v, and ly indicates microscopic invasion or metastasis to anterior capsule, retroperitoneal tissues, lymph node, vein, and lymphatic vessels, respectively, and the extent of tumor invasion or metastasis is expressed as numerical order. Tumor size (size) and stage are assigned according to the extent of tumor invasion.

K-ras mutation in portal blood

case	pathol	size	s	rp	n	v	v	ly	stage	K-ras	
										pre	post
(1)	tub2	t2	1	3	1	2	2	2	IVa	−	−
(2)	tub2	t3	1	1	1	3	3	3	IVa	−	−
(3)	tub2	t3	3	0	1	2	2	1	IVa	−	−
(4)	tub2	t2	1	1	1	0	0	1	III	ND	+
(5)	tub2	t2	0	1	1	1	1	1	III	−	−
(6)	tub2	t3	0	2	1	1	1	1	IVa	−	−
(7)	tub2	t2	1	1	1	1	1	1	III	−	+
(8)	tub2	t3	1	1	2	2	2	2	IVb	ND	+
(9)	tub2	tla	0	0	1	1	1	1	II	−	−

none of the patients were positive at the beginning of surgery.

Discussion

The formation of a metastasis involves the transformation of normal cells into tumor cells and the progressive growth of neoplastic cells, extensive vascularization, local invasion of the host stroma, detachment and embolization, survival in the circulation, arrest in the capillary endothelial cells of distant organs, extravasation, and proliferation within the organ parenchyma[12]. These steps need to occur in a time-limiting and sequential interrelated manner to complete the metastatic process, which means that failure at any stage aborts the whole process. Most tumor cells released in the blood stream are rapidly eliminated by blood turbulance, deformability deficiency, and lysis mediated by endothelial cells; therefore, the mere presence of tumor cells in the circulation does not predict that metastasis will occur[13]. Fidler[14] demonstrated that less than 1 % of tumor cells placed are viable 24 hours after entry into the circulation, and less than 0.1% eventually survive to produce metastasis. Thus, the probability of the establishment of metastasis seems to depend to some degree on the number of viable cells in the blood stream. In the present study, K-ras mutations were detected in three of nine patients with pancreatic carcinoma. K-ras mutations do not always determine the presence of viable cancer cells in the portal blood, because non-viable cell fragments from tumor cells with K-ras mutation can result in the positive status of K-ras mutation by a highly sensitive PCR method. The presence of K-ras mutations in the portal blood is indicative of an increased potential to develop liver metastasis, however, no liver metstasis developed in three positive cases to this stage. The sensitivities of K-ras mutations differ from the methods[7,15] employed for the detection. Mutation alleles specific amplification method employed in this study can detect K-ras mutation if more than 0.5% of total DNAs are mutated. Blood samples contain the large amount of lymphoid cells, which occupy the large part of the sample DNAs examined in PCR. The more the negative cells exsist in the sample, the less the detectability of the positive cells becomes. Regarding the optimum timing for sample

collection, while K-ras mutations were detected at the time of resection in some patients, none were positive at the time of laparotomy, indicating the possibility that surgery might promote the migration of tumor cells into the portal blood. In addition to complete surgical resection or irradiation to the retroperitoneum for the control of the local disease, intra- and postoperative chemotherapy to combat liver metastases may be necessary to improve the outcome[16]. Tada et al[7]. reported that K-ras mutation was detected in the peripheral blood and pancreatic juice of two patients among six with pancreatic adenocarcinoma, four of whom had advanced unresectable adenocarcinoma with metastases to remote organs. According to recent reports, epithelial tumor cells have been found in bone marrow taken from patients with pancreatic carcinoma before any treatment, by immunocytochemical detection using monoclonal antibodies directed to epithelial cytokeratin[17]. Moreover, the occurrence of tumor relapse after complete surgical resection was observed to be significantly associated with cytokeratin-positivity in bone marrow. Therefore, effective systemic neoadjuvant therapy should be established to control or eliminate occult metastases to promote the success of pancreatic cancer treatment. It is speculated that cancer cells have the ability to metastasize in the process of malignant transformation and that some genetic abnormalities are responsible for the acquisition of metastatic ability. K-ras mutation is thought to be an early event in the carcinogenesis of pancreatic cancer[18], and is supposed to correlate with the occurrence of metastasis in colorectal cancer[19]. A variant CD44, which is highly expressed in human pancreatic adenocarcinoma[20], has been shown to confer the metastatic potential to the carcinoma cells[21]. Interestingly, treatment using the monoclonal anti-CD44 antibody retarded metastatic tumor growth in rats[22]. A close link between metastatic behavior and CD44 promoter activity is induced by ras[23]. Thus, a high prevalence of K-ras mutation might determine strong metastatic activity of pancreatic adenocarcinoma.

A previous clinicopathological study of resected specimens revealed that recurrences of pancreatic cancer in the liver within 1 year after surgery was associated with the presence of venous and lymphatic invasion[23] ; however, K-ras mutation was not always detected in cases of massive venous or lymphatic invasion.

In conclusion, K-ras mutations were identified in portal blood taken during surgery in some patients with pancreatic adenocarcinoma. Further study is necessary to clarify whether resection might promote the migration of cancer cells into the portal vein, and if the presence of K-ras mutation in the portal blood can predict the postoperative reccurrence of pancreatic cancer in the liver.

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