Follow-Up Study of K-*ras* Mutations in the Plasma of Patients With Pancreatic Cancer

Correlation With Clinical Features and Carbohydrate Antigen 19-9

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Objective: We followed up the presence of Kirsten rat sarcoma (K-*ras*) mutations in plasma DNA and assessed its clinical value in patients with pancreatic cancer.

Methods: Plasma samples (N = 430) of 56 patients with pancreatic cancer and 13 patients with pancreatitis were analyzed by real-time polymerase chain reaction using peptide nucleic acid–mediated polymerase chain reaction clamping.

Results: K-*ras* mutations could be detected in the plasma DNA of 20 patients with cancer (36%). No K-*ras* mutation was found in the plasma of patients with pancreatitis. In 7 (35%) of 20 patients with lowly or moderately elevated carbohydrate antigen 19.9 (CA 19-9) levels lower than 100 U/mL, the result of the assay was positive for K-*ras* mutation. The combination of K-*ras* and CA 19-9 level determination gave a sensitivity for the diagnosis of pancreatic cancer of 91% (40/44) of the patients. Thirteen of 35 patients with pancreatic cancer (102 plasma samples) with elevated CA 19-9 levels (>35 U/mL) and altered K-*ras* gene showed significant correlation with elevated CA 19-9 levels ($P = 0.048$).

Conclusions: The summary of our approach of noninvasive, convenient, extremely high-sensitive K-*ras* mutation analysis in plasma might provide diagnostic and prognostic information to clinicians but will not be sufficient in a standardized early diagnosis of pancreatic carcinoma. The combination with CA 19-9 assay is useful for detection and prognostic evaluation of pancreatic carcinoma.

Key Words: pancreas, tumor, K-*ras*, plasma, PNA, CA 19-9

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Pancreatic cancer is a major cause of death in Western populations and has a poor prognosis. The median survival after diagnosis is shorter than 6 months, and 5-year survival is 3% to 5%.[1,2] Surgical resection is the only treatment offering patients with pancreatic cancer cure, but the resectability rate remains at 15% to 20% because the tumor is frequently beyond surgical resectability or has already metastasized when symptoms first appear.[3] The current postresection 5-year survival rate is approximately 20%.[4]

Pancreatic cancer is among the best-described genetic diseases. Accumulated genetic alterations in dominant oncogenes, such as Kirsten rat sarcoma (K-*ras*), and tumor suppressor genes, such as p53, p16 (cyclin-dependent kinase inhibitor 2 [*CDKN2*]), and *DPC4* (*SMAD4* or *MADH4*), are involved in the pathogenesis of pancreatic cancer.[3,5] The K-*ras* oncogene is mutated (almost always confined to codon 12) in 75% to 95% of exocrine pancreatic cancers.[6] K-*ras* mutations provoke an inappropriate stimulation signal and a constitutive growth signal to the nucleus. Alterations of the K-*ras* gene can be detected in clinical specimens (tissue samples, pancreatic juice, duodenal aspirates, stool, and serum) by different methods such as restriction fragment length polymorphism, single-strand confirmation polymorphism, mutant-specific allele amplification, sequencing, or selective hybridization.[4]

Although there have been advances in imaging techniques, diagnosing the disease remains difficult, succeeding normally only in advanced stages of disease. Conventional tumor markers, such as serum carbohydrate antigen 19.9 (CA 19-9), have inadequate diagnostic sensitivity and specificity and are therefore not suited for detecting early-stage tumors.[4] Different studies have reported that plasma K-*ras* mutation analysis can complete the serum CA 19-9 level determination, provide additional clinically useful information, and detect most cases of pancreatic cancer.[7,8] A noninvasive prognostic tool with a very high sensitivity and specificity could be extremely helpful to improve the poor prognosis of patients with pancreatic cancer.

According to previous studies, we investigated the presence of K-*ras* mutations in circulating plasma DNA and assessed its clinical value in patients with pancreatic cancer. Peripheral blood samples of a large series of patients with pancreatic cancer and of controls with pancreatitis were analyzed. We determined the occurrence of K-*ras* gene alterations in a follow-up study for a period of approximately 2 years (if available). In particular, we investigated the diagnostic value of combined K-*ras* mutations and serum CA 19-9 levels in patients with pancreatic carcinoma. The detection of K-*ras* mutations was performed by using the highly sensitive method of the polymerase chain reaction (PCR) clamping approach with melting curve analysis using mutant-specific hybridization probes combined with wild type–specific peptide nucleic acids (PNAs).[9] All in all, the aim was to evaluate the prognostic value of the K-*ras* status in the peripheral blood of patients with pancreatic cancer.

MATERIALS AND METHODS

Patients

This study included 56 patients with pancreatic cancer. The median age at initial diagnosis was 59.7 years, and the

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The authors of the manuscript affirm that patient informed consent was obtained.

The authors made the following contributions for the completion of the study: guarantor of the article, J.D.; study design, J.D., R.P., J.H., and H.O.; research, data, and statistical analysis, J.D. and R.P.; and manuscript preparation, J.D., R.P., and J.H.

male-female ratio was 1.2:1 (31 men and 25 women). Patients were followed up in the period (as possible) from diagnosis to the end of the observation period or death. Twenty-five patients underwent surgical resection, and 23 patients were primarily inoperable (the data of the remaining 8 patients were not available). The median survival of the total patient population (51 patients with known date of initial diagnosis) was 19 months. The median survival of the patients with operable disease was 28 months, whereas the patients with advanced/unresectable pancreatic cancer survived for a median of only 10 months. At the end of the study, all but 5 patients with pancreatic cancer were dead.

A control group of 13 patients with chronic or acute pancreatitis were recruited and included. We collected 1 blood sample from each control patient and performed the K-*ras* assay.

Plasma DNA

Peripheral venous blood (9 mL) was taken and placed in tubes containing EDTA after informed consent was obtained from the patients and the controls. Samples were taken from the patients with pancreatic cancer (as possible) every 4 weeks after initial diagnosis until the end of the observation period or death. Four hundred seventeen plasma samples of 56 patients with pancreatic cancer and 13 plasma samples of 13 patients with pancreatitis (controls) were collected for analysis of K-*ras* mutations. Blood samples were centrifuged at 1500 rpm for 10 minutes (Minifuge RF; Heraeus, Hanau, Germany). Plasma was stored at −20°C until further use. DNA was extracted from plasma with QIAamp spin columns (QIAamp DNA Mini Kit; Qiagen, Hilden, Germany) according to the manufacturer's instruction. Incubation with proteinase K was done for 10 minutes at 68°C. Extracted DNA from 200 μL of plasma was eluted with 50 μL and stored at −20°C.

For the determination of CA 19-9 levels, we collected 239 serum samples from 44 of the included patients with pancreatic cancer at the same time as the collection of plasma for K-*ras* analysis. We correlated the results from the 239 matched samples to determine any relationship between K-*ras* status and CA 19-9 serum level.

Detection of K-*ras* Gene Mutations

For detection of the hot spot point mutation of the K-*ras* proto-oncogene at codon 12, we used PNA-mediated PCR clamping and real-time PCR with mutant-specific hybridization probes. The relative positions of the primers, hybridization probes, and PNA, as well as the high sensitivity (1:100,000, mutant–wild type), of our assay were described elsewhere (GenBank accession No. K01519; nucleotide positions 1–164).[9] Real-time PCR was performed at a final volume of 20 μL containing 10 mmol/L Tris-HCl with a pH of 8.3, 50 mmol/L of KCl, 3.75 mmol/L of $MgCl_2$, 125 μmol/L of each desoxynucleotide triphospate (Invitrogen, Carlsbad, Calif), 1 μmol/L of sense and antisense primers, 0.3 μmol/L of hybridization probe K-*ras* FL (donor) labeled on its 3′-end with fluorescein, and 0.3 μmol/L of K-*ras* LC (acceptor) labeled on its 5′-end with the fluorescence dye LightCycler-Red, 2.5 μmol/L of PNA 17-mer, Protein AmpliFLY, and 1.25 U of Platinum Taq DNA Polymerase (Invitrogen). After an initial denaturation step at 95°C for 3 minutes, 45 cycles were performed, with each cycle involving denaturation at 95°C for 10 seconds, PNA annealing at 76°C for 7 seconds, annealing of the primers and probes at 60°C for 15 seconds, and elongation at 72°C for 20 seconds. The PCRs were carried out on the LightCycler Instrument (Roche Diagnostics, Mannheim, Germany).

Melting curve analysis was performed at steadily increasing temperature from 40°C to 85°C with a transition rate of 0.3°C/s. Fluorescence data obtained were analyzed using the LightCycler software (version 3.5; Roche Diagnostics). All synthetic oligonucleotides were purchased from TIB Molbiol (Berlin, Germany).

The PCR experiments were performed at least twice on each sample. The DNA of the cell line SW480 (colon carcinoma) harboring the homozygous K-*ras* codon 12 GTT mutation was used as a positive control.

Statistical Analysis

Statistical comparisons of the different data between groups (with results positive vs negative for K-*ras* mutation in plasma) were tested by *t* test, χ^2 test, Monte Carlo exact test, and log-rank test. The Mann-Whitney-Wilcoxon *U* test was used to correlate the K-*ras* status in peripheral blood with the serum concentration of CA 19-9. Differences were considered significant when $P < 0.05$. All calculations were performed by using the Statistical Package for the Social Sciences (SPSS Inc, version 11.0, Chicago, Ill).

RESULTS

K-*ras* Gene Mutations in Plasma

Adequate DNA was extracted from the plasma in sufficient quantities for analysis in all patients and controls. The concentrations of DNA extracted from the plasma of patients with pancreatic cancer and pancreatitis were determined by spectrophotometry and ranged from 100 to 700 pg per 200 μL of plasma.

Correlation of K-*ras* Gene Alteration and Clinical Features

K-*ras* mutations were identified in 20 (36%) of 56 patients with pancreatic cancer and 60 (14%) of 417 plasma samples (Table 1). We observed alternating results of the K-*ras* assay in individual patients with cancer during follow-up. Thereby, it is remarkable that K-*ras* mutations were particularly detected shortly after initial diagnosis and/or before death (Table 1, patients 5, 8–10, 17, 27, 34, 39, 43, 44, and 49). In the intermediate time interval, the detection of K-*ras* mutations in the peripheral blood was negative (patients 5, 17, 34, 44, and 49) or predominantly persistent (patients 8, 9, 11, and 43). Other patients (3, 6, 24, 28, 35, 36, 51, and 56) showed isolated positive results for the detection of K-*ras* mutations in the peripheral blood. The most common alteration in the detectable plasma K-*ras* mutations (16 of 20 patients) was from wild type GGT (encoding glycine) to GTT (valine). The remaining 4 patients showed a transition different from GTT (nonvaline). Further verification of these point mutations was not done. The DNA from the plasma samples of the control group of 13 patients with chronic or acute pancreatitis were analyzed for K-*ras* mutations. In 12 patients, no gene alterations were detectable, and for 1 patient, the result was ambiguous (data not shown). The positive predictive value for plasma K-*ras* mutation detection was 100%, and the negative predictive value was 27%. There were no statistically significant differences in age ($P = 0.58$), sex ($P = 0.28$), localization of the tumor in the pancreas ($P = 1.00$), resectability of the tumor ($P = 0.33$), and survival ($P = 0.10$) according to the presence or absence of plasma K-*ras* mutations (Table 2).

Plasma K-*ras* Mutations and CA 19-9

Elevated CA 19-9 levels were found in 35 (80%) of the 44 patients using a cutoff value higher than 37 U/mL and in

TABLE 1. Analyzed Plasma Samples

	FS	Months After First Sample																						
No.	**Ex**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
1	3	WT	NA	NA	WT	NA	NA	WT	WT	NA	NA	← K-*ras* status in plasma												
	10	NA			4539			9648	NA			← CA 19-9 level in serum, U/mL												
2	1	WT	WT	WT	WT	WT	WT	NA	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	NA	⋙		NA
	26	7	7	4	10	20	50		NA	NA	NA	NA	NA	137	106	103	177	137	286	225				
3	1	WT	NA	NA	NA	**Mut**	WT	WT	WT	WT	WT	**Mut**	WT	WT	NA			⋙			NA	WT	WT	NA
	28	4282	161			22	71	213	NA	1313	NA	4794	$>10^4$	NA							$>10^4$	NA	NA	
4	9	WT	NA	NA	NA	WT	NA	WT	WT	NA	WT	NA	WT	NA	NA	NA	NA							
	16	13				14		15	17		25		33											
5	15	**Val**	NA	WT	WT	NA	WT	NA	NA	NA	NA	NA	**Val**	**Val**	**Val**	**Val**	**Val**	**Val**	**Val**	**Val**				
	19	NA		4	<1	<1	NA						7	NA	NA	NA	NA	4	17	15				
6	13	WT	NA	NA	**Val**	WT	NA	WT	WT	WT	WT	WT	WT	WT	NA			⋙			WT	NA	⋙	NA
	35	82			49	62		77	97	83	NA	98	75	NA							NA			
7	0	WT	WT	WT	WT	NA																		
	5	$>10^5$	NA	$>10^5$	$>10^6$																			
8	2	**Val**	NA	WT	**Val**	NA	WT	WT	**Val**	WT	**Val**	**Val**	**Val**	WT	NA	NA	WT	WT	NA					
	18	NA		NA	NA		NA	NA	NA	NA	NA	NA	NA	6058			4633	698						
9	0	**Val**	NA	WT	**Val**	**Val**	WT	NA	**Val**	NA	**Val**	**Val**	NA	**Val**	NA	WT	NA	WT	NA	WT	NA	⋙		NA
	30	NA		NA	NA	NA	NA		NA		NA	NA		NA		2		<1		5				
10	3	**Val**																						
	2	$>10^4$																						
11	3	WT	**Val**	**Val**	NA	**Val**	**Val**	**Val**	**Val**															
	8	2654	2706	5288		$>10^4$	8077	NA	$>10^4$															
12	10	WT	NA	WT	NA	WT	NA	WT	NA	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
	30	11		11		22		44		83	203	490	NA	NA	NA	115	295	1296	1466	1852	2111	1785	2086	NA
13	0	WT	NA	WT	NA	WT	WT	WT																
	7	6879		698	654	2254	$>10^4$	$>10^4$																
14	21	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	NA	WT
	32	35	30	30	34	NA	45	41	50	NA	61	60	NA	NA	NA	NA	133	126	176	NA	211	182		181
15	8	WT	NA																					
	2	NA																						
16	22	WT	NA	WT	NA	WT	NA	WT	WT	WT	WT	WT	NA	WT	WT									
	14	109	62	43		45	90	NA	191	340	426	1948		4216	NA									
17	2	**Mut**	NA	NA	WT	WT	WT	WT	WT	WT	WT	NA	NA	NA	NA	NA								
	15	NA			493	473	559	NA	260	NA	NA				$>10^4$									
18	4	WT	NA	NA	NA	WT	NA	NA																
	7	125				1516																		
19	3	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	NA	WT	WT	WT	WT	WT	WT	NA
	32	8	6	NA	8	9	NA	NA	NA	15	NA	15	NA	NA	NA	14		12	28	30	32	39	80	

20	2	WT	WT	WT	NA	NA	NA																			
	7	7	2	NA																						
21	2	WT	NA	NA																						
	3	NA																								
22	12	WT	NA	WT	WT	WT	NA																			
	7	NA	NA	NA	NA	NA	74																			
23	NA	WT	WT	NA																						
	3	NA	NA																							
24	2	WT	NA	NA	WT	WT	NA	WT	**Val**	WT	WT	WT	NA	WT	WT	NA										
	17	NA			509	621		633	552	592	552	803	949	NA	1313	NA										
25	0	WT	WT																							
	2	$>10^5$	$>10^5$																							
26	1	NA	NA	WT	NA	WT	WT	NA	NA	NA																
	9	138	142	95		152	278																			
27	21	**Val**	NA	NA																						
	3	$>10^4$																								
28	**Val**	NA	WT	WT	WT	NA	WT	**Val**	NA	**Val**	WT	WT	WT													
	14		NA	NA	NA		NA	39		NA	NA	NA	NA													
29	2	WT	WT	WT	NA																					
	4	1	NA	9	32																					
30	2	WT	WT	NA	WT	WT	NA																			
	6	2164	2111		4657	7651	$>10^4$																			
31	16	WT	NA	NA	WT	NA	WT	WT	WT	NA	WT	WT	NA													
	13	302		183	NA		91	99	NA	294	NA	578														
32	0	WT	NA	NA	NA	WT	NA	NA	NA	NA	NA	NA														
	11	NA				NA																				
33	3	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	NA	WT	WT	WT								
	20	9156	7953	2730	1211	NA	3748	NA	3861	NA	$>10^4$			2876	2729	NA	6626	8808								
34	1	WT	NA	WT	WT	**Mut**	**Mut**	**Mut**																		
	7	5916		2291	4169	$>10^4$	$>10^4$	$>10^5$																		
35	2	WT	WT	WT	WT	**Val**	WT	WT	WT	WT	WT	WT	WT	WT	WT	NA	NA									
	15	123	464	1810	597	517	546	1148	NA	NA	NA	NA	NA	NA												
36	1	WT	NA	NA	**Val**	WT	WT	NA																		
	7	3			<1	<1	2																			
37	11	WT	NA	WT	NA	WT	WT	WT	WT	WT	WT	WT	NA	NA												
	13	7	NA	13		28	37	NA	41	50	52	56														
38	1	WT	WT	WT	WT																					
	5	$>10^6$	$>10^6$	NA	$>10^6$																					
39	82	WT	**Val**	NA	NA																					
	4	$>10^4$	$>10^4$																							
40	31	WT	NA	NA	WT	NA	WT	WT	WT	WT	WT	WT	NA	WT												
	13	NA		309	NA		464	NA	179	NA	NA	218		251												

(Continued next page)

TABLE 1. *(Continued)*

	FS	Months After First Sample																					
No.	**Ex**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
41	1	WT	WT	WT	WT	WT																		
	5	$>10^4$	$>10^4$	$>10^4$	8850	$>10^4$																		
42	NA	WT	WT	NA																				
	3	NA	NA																					
43	1	**Val**	NA	NA	**Val**	**Val**	**Val**	**Val**	**Val**	**Val**	**Val**	**Val**	NA	NA										
	13	677		434	1365	2116	5231	883	446	544	1506	$>10^4$	$>10^4$											
44	7	**Val**	**Val**	WT	WT	WT	WT	WT	WT	NA	WT	WT	WT	NA	**Val**	NA	NA	NA	NA	NA				
	19	NA	NA	NA	NA	NA	NA	NA	NA		NA	NA	NA	5	8	10								
45	NA	WT	WT	WT	WT																			
	4	NA	NA	NA	NA																			
46	10	WT	NA	NA	NA	WT	WT	NA	NA	NA	WT	NA	NA	NA	WT	NA	NA	NA	NA	NA	NA	NA	WT	NA
	30	14				15	NA				8						11	15	10		14		14	
47	1	WT	WT	NA	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT								
	15	$>10^4$	$>10^4$		NA	$>10^5$	$>10^4$	NA	$>10^4$	$>10^4$	$>10^4$	$>10^4$	$>10^4$	$>10^5$	NA	$>10^5$								
48	25	WT	WT	WT	WT	WT	WT	NA	WT	NA	NA													
	10	NA	NA	NA	NA	2281	NA	1368	NA															
49	5	**Val**	NA	NA	WT	NA	**Val**	NA	WT	NA	WT	NA	WT	NA	WT	NA	WT	NA	NA	WT	WT	WT	WT	NA
	33	47			68		124		146		151		112		NA		NA			230	283	389	660	1046
50	NA	WT	WT	NA	WT	NA	NA																	
	6	NA	NA		NA																			
51	5	WT	WT	NA	WT	WT	**Val**	NA	WT	WT	WT	WT	NA											
	12	NA	NA		NA	NA	10		NA	NA	NA	NA												
52	13	WT	NA	WT	WT	WT	WT	NA	WT	NA	WT	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		
	21	NA		NA	NA	NA	NA		NA		NA													
53	12	WT	WT	WT	WT	WT	WT	WT	NA	NA	NA	WT												
	11	NA	NA	NA	NA	NA	NA	NA				NA												
54	18	WT	NA	NA	NA	WT	WT	NA	NA	NA	NA	NA	NA											
	12	NA				NA	NA																	
55	2	WT	WT	NA	WT	NA	WT	WT	WT	WT	WT	WT	WT											
	12	NA	NA		NA		NA	NA	NA	NA	NA	NA	NA											
56	20	**Mut**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA										
	13	NA																						

K-*ras* mutations (Val/Mut) are in bold.

Ex indicates death in months after collection of the first sample; FS, first collected sample after diagnosis (in months); Mut, mutation different from valine; NA, no data available; Val, valine (mutation); WT, wild type (glycine); ⋙, bigger time interval than indicated in the time line.

TABLE 2. Clinical Features From Patients With Pancreatic Cancer With Positive and Negative Detection of K-*ras* Mutation in Plasma DNA

K-*ras* Mutation	Positive (n = 20)	Negative (n = 36)	*P*
Age	58.6	60.3	0.575*
Male-female	13:7	18:18	0.279†
Tumor site			1.000‡
Head	13	25	
Tail	1	3	
Papilla	0	1	
No data	6	7	
Resectability			0.332†
Inoperable	7	16	
Operable	11	14	
No data	2	6	
CA 19-9 level			0.402†
>37 U/mL	13	22	
≤37 U/mL	5	4	
No data	2	10	
Survival time			
Median, mo	20	14	0.099§

*t Test.
†χ^2 test.
‡Monte Carlo exact test.
§Log-rank test.

30 (68%) of the 44 patients using a cutoff value higher than 100 U/mL. No CA 19-9 values of the patients with pancreatitis used as the control group for the K-*ras* assay were available.

In 7 (35%) of 20 patients with pancreatic cancer or 10 of 86 plasma samples with CA 19-9 levels lower than 100 U/mL, the result of the assay was positive for K-*ras* mutation. Five of the 7 patients with K-*ras* variants and CA 19-9 levels lower than 100 U/mL had CA 19-9 levels lower than 37 U/mL. Elevation of either CA 19-9 level (cutoff, >37 U/mL) or the presence of K-*ras* mutations in plasma was seen in 40 (91%) of the 44 patients. Of the 35 patients with pancreatic cancer and elevated serum levels of the tumor marker CA 19-9 (cutoff, >37 U/mL), 13 (102 plasma samples) with altered K-*ras* gene showed significant correlation with elevated CA 19-9 levels (median, 552 U/mL; $P = 0.048$; Fig. 1).

DISCUSSION

In the present study, one of the most frequent K-*ras* gene mutation (valine) observed in pancreatic cancer was analyzed in the plasma of a patient with pancreatic cancer and pancreatitis (controls). Whereas previous studies analyzed 1 sample per individual after diagnosis of pancreatic cancer, we present a study of time course analysis of K-*ras* gene alterations. The occurrence or absence of K-*ras* mutations in the peripheral blood might reflect different tumor stages in 1 patient, depending on the time of observation. Therefore, it is anticipated that there are patients who tested positive for the K-*ras* mutation in some plasma samples and negative in others. Indeed, we observed alternating results of the K-*ras*

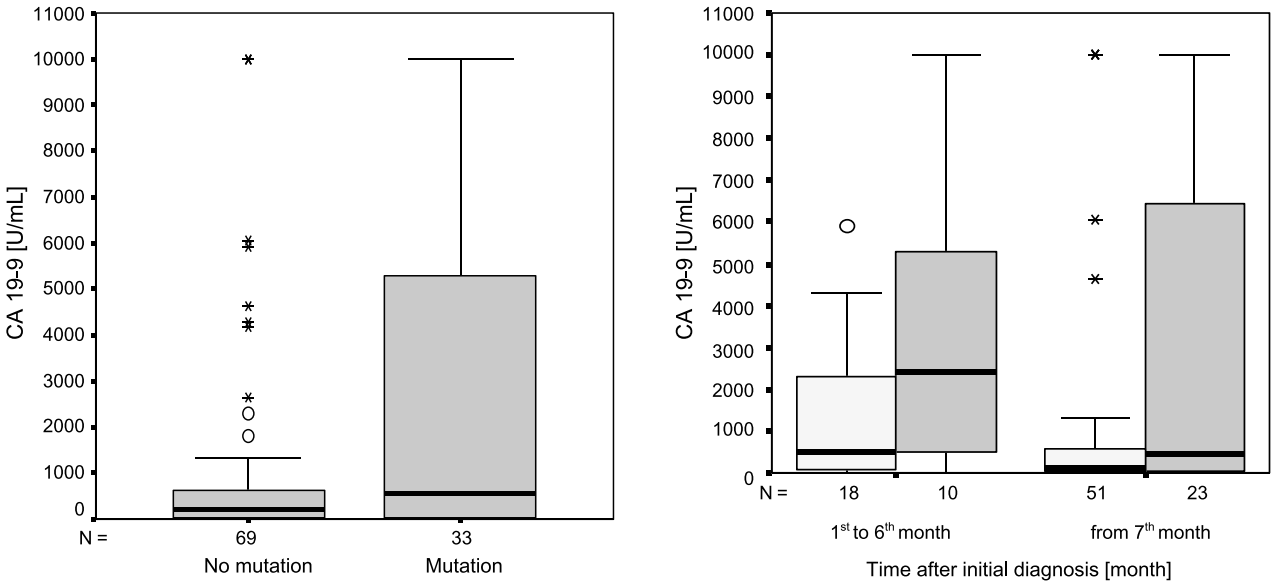

FIGURE 1. Plasma samples (n = 102) of 13 patients with pancreatic cancer with elevated serum levels of the tumor marker CA 19-9 (cutoff, >37 U/mL) and detected K-*ras* gene alteration in the peripheral blood at least once during the observation period were analyzed regarding correlation between K-*ras* status and CA 19-9 plasma level. Positive detection of K-*ras* mutation showed significant correlation with elevated CA 19-9 levels (median, 552 U/mL; $P = 0.048$). Illustrated is the total observation period (left) and, in addition, 2 time slices (right, dark gray, mutation; light gray, wild type). Outliers are indicated by open dots and asterisks. "Extreme" outliers, or those which lie more than 3 times the interquartile range (IQR) above and below of the first and third quartiles respectively, are indicated by the presence of an asterisk. "Mild" outliers, or those which lie more than 1.5 times the IQR above and below the first and third quartile but are not also extreme outliers are indicated by the presence of an open dot.

assay in individual patients with cancer, with higher incidence after initial diagnosis and before death. These findings indicate that the detection of K-*ras* mutations in the peripheral blood could reflect the tumor burden of individual patients with pancreatic cancer. Otherwise, isolated positive results for the detection of K-*ras* mutations in the peripheral blood during follow-up could be a sign of transient hematogenous tumor cell dissemination. However, our study was not designed to investigate the correlation of the detection of K-*ras* mutations and medical (chemo-)therapies, the results of different imaging modalities, and/or other clinical parameters at consecutive points of time.

Detection of K-*ras* mutations in circulating DNA had a low sensitivity (36%) but a high specificity (100%) for the diagnosis of pancreatic cancer. The sensitivity was in agreement with previous studies (27%–81%).[1,5–8,10–17] Higher K-*ras* mutation prevalences have been reported in pancreatic or duodenal juice (63% to 100%) probably owing to the higher amount of tumor DNA in those specimens as compared with the plasma samples.[12,18,19–24] When combined with CA 19-9, sensitivity was raised to 91% (discussed later). K-*ras* mutations have been reported in pancreatic tissue or juice from 6% to 42% of patients with chronic pancreatitis.[6] Knowing that a part of this mutated DNA can be released into circulation, our control group included patients with pancreatitis.[7] Even so, we revealed a specificity of 100%. The specificity in other analogue studies ranged from 87% to 100%, depending on the quantity and quality of the control group.[1,6,10,16,17]

Serum CA 19-9 is one of the most commonly used tumor markers for pancreatic cancer diagnosis, with sensitivity in the range of 58% to 87%.[4] This marker is not informative in 5% of the population who cannot express CA 19-9 because of a Lewis negative status.[25] However, serum CA 19-9 lacks specificity (70%–80%).[6] Serum CA 19-9 levels may also be raised in other malignancies, such as adenocarcinoma of the stomach, colon, and the hepatobiliary system, and in obstructive jaundice, acute cholangitis, and worst of all, in chronic pancreatitis.[26] In the present study, serum CA 19-9 had good sensitivity (80%). The incidence of 35% (7/20 patients) of positive K-*ras* detection in plasma at a time of nonelevated CA 19-9 levels suggests that the analysis of the K-*ras* status can identify patients with pancreatic cancer who have lower CA 19-9 levels than are usually associated with this cancer. Other studies show an even higher incidence of 71% (25/35 patients): Yamada et al,[7] 3 of 6 patients; Dianxu et al,[8] 12 of 17 patients; Theodor et al,[10] 6 of 7 patients; and Sorenson et al,[14] 4 of 5 patients. The absence of K-*ras* mutations and the abnormal CA 19-9 levels made the diagnosis of pancreatic cancer unlikely. Thus, the combination of both tests, that is, the detection of K-*ras* alterations in the DNA of peripheral blood and the determination of CA 19-9 level, could be useful to assess cancer diagnosis in patients with normal or, because of individual genetic alterations for Lewis enzyme expression, noncontributive CA 19-9 levels and also to provide additional supportive information.

In the present study, the occurrence of K-*ras* mutations in peripheral blood was not correlated with age, sex, tumor site, resectability of the tumor, and survival. These results are in agreement with other studies. Uemera et al[5] found no associations between K-*ras* mutations in the plasma DNA and age, sex, size of the tumor, stage of disease, or presence of metastasis. Maire et al[6] found that the presence of K-*ras* mutations in serum was not correlated with age, sex, smoking habit, or tumor stage. Three other studies in the literature are in agreement with these results, but Castells et al[1] have reported a statistically significant relation between circulating DNA K-*ras* alterations and survival time.[7,8,10]

Early diagnosis of pancreatic carcinoma is extremely difficult. Plasma DNA is a reliable source for K-*ras* gene mutation analysis. Our approach of noninvasive, convenient, extremely highly sensitive K-*ras* mutation analysis in plasma might provide diagnostic and prognostic information to clinicians. The combination with CA 19-9 assay is useful for the detection and prognostic evaluation of pancreatic carcinoma. Larger studies are required to assess the significance of our findings to clinical practice.

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