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Peripheral and Portal Venous *KRAS* ctDNA Detection as Independent Prognostic Markers of Early Tumor Recurrence in Pancreatic Ductal Adenocarcinoma

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BACKGROUND: *KRAS* circulating tumor DNA (ctDNA) has shown biomarker potential for pancreatic ductal adenocarcinoma (PDAC) but has not been applied in clinical routine yet. We aim to improve clinical applicability of ctDNA detection in PDAC and to study the impact of blood-draw site and time point on the detectability and prognostic role of *KRAS* mutations.

METHODS: 221 blood samples from 108 PDAC patients (65 curative, 43 palliative) were analyzed. Baseline peripheral and tumor-draining portal venous (PV), postoperative, and follow-up blood were analyzed and correlated with prognosis.

RESULTS: Significantly higher *KRAS* mutant detection rates and copy numbers were observed in palliative compared to curative patients baseline blood (58.1% vs 24.6%; $P = 0.002$; and $P < 0.001$). Significantly higher *KRAS* mutant copies were found in PV blood compared to baseline ($P < 0.05$) samples. KRAS detection in pre- and postoperative and PV blood were significantly associated with shorter recurrence-free survival (all $P < 0.015$) and identified as independent prognostic markers. *KRAS* ctDNA status was also an independent unfavorable prognostic factor for shorter overall survival in both palliative and curative cohorts (hazard ratio [HR] 4.9, $P = 0.011$; HR 6.9, $P = 0.008$).

CONCLUSIONS: *KRAS* ctDNA detection is an independent adverse prognostic marker in curative and palliative PDAC patients—at all sites of blood draw and a strong follow-up marker. The most substantial prognostic impact was seen for PV blood, which could be an effective novel tool for identifying prognostic borderline patients —guiding future decision-making on neoadjuvant treatment despite anatomical resectability. In addition, higher PV mutant copy numbers contribute to an improved technical feasibility.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the European Union (1). Due to unspecific or lack of symptoms, PDAC is diagnosed >80% at an advanced stage with an overall 5-year survival rate of 10.8% (2, 3).

Patients resected at an early disease stage, have a 5-year overall survival (OS) of <40%, reflecting the high incidence of occult metastasis—which is one main factor for dismal prognosis in a potential curative setting (2). Biomarkers that serve as a surrogate for occult metastasis are needed to improve the selection of patients who will most likely profit from neoadjuvant treatment despite anatomical resectability.

The current guidelines recommend evaluating neoadjuvant treatment in patients with prognostic borderline characteristics (high Ca 19-9 levels, proven nodal positivity) (4). The available evidence for the Ca 19-9 cutoff level is mainly based on small or moderate-sized patient cohorts, and detection of nodal positivity requires

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a PET-CT, which is currently not standard for PDAC staging (5–7). Therefore, searching for additional cost-efficient and reliable biomarkers to identify prognostic borderline patients is critical since these patients should receive neoadjuvant treatment despite anatomical resectability due to the high risk of occult metastasis (8).

Circulating tumor DNA (ctDNA) might improve the identification of prognostic borderline PDAC patients. Liquid biopsy, the analysis of tumor-related material in the blood and other patient-derived body fluids, has emerged as a novel, minimally invasive strategy to diagnose cancer patients and monitor disease progression (9). Circulating cell-free DNA (cfDNA) is released into the bloodstream by both healthy and cancerous cells undergoing apoptosis and necrosis. The portion of cfDNA that is released by tumor cells, the ctDNA, only makes up a small percentage of around 0.01–5% of the total cfDNA (10). Sensitive methods of detection are thus necessary to detect small amounts of ctDNA. Digital droplet PCR (ddPCR) has become one of the most widely used methodologies for detecting small amounts of tumor DNA because of its high sensitivity and accuracy (11–13). To differentiate ctDNA from cfDNA, one must target specific mutations present in the tumor. KRAS is a driver gene in PDAC and mutated in about 80–90% of patients. The mutations are concentrated in a few "mutation hotspots" on codons 12 and 61, making it an ideal target for ddPCR-based detection assays (14). KRAS-based mutation detection has been used in several ctDNA studies in PDAC with prognostic relevance (15–17). The ctDNA technology shows great potential to optimize individualized therapy in PDAC patients but still needs to prove its clinical value to be finally translated into daily clinical routine.

Our study compared intraoperative portal vein punctures with peripheral blood before and after surgery to study the value of tumor-draining PV blood as a novel liquid biopsy source for KRAS mutation detection in PDAC. The presence of ctDNA and the number of mutant copies at different time points and locations were used for survival analyses to test the hypothesis that ctDNA might be a biomarker for a dismal prognosis in general and especially for those patients with high risk of early relapse after surgery, who might benefit from neoadjuvant treatment, independent of anatomical resectability.

Materials and Methods

STUDY COHORT

The local ethical committee approved the study, and written informed consent was obtained from all patients. The study has been registered in the German Clinical Trials Register (DRKS00023362).

For our prospective explorative study, 108 adult patients with confirmed PDAC histology were included between October 2019 and December 2021 (Supplemental Fig. 1). Of these, 43 patients were in the palliative cohort (undergoing palliative systemic treatment only [UICC III and IV]), and 65 were in the curative study cohort (undergoing curative surgery and chemotherapy [UICC I-III]) (Table 1). Patients with secondary neoplasia were excluded. Clinical follow-up data with a median follow-up time of 11 months data on treatment, tumor recurrence, and death were obtained.

We collected peripheral blood at baseline (before primary surgery, start of systemic treatment, or after diagnosis) from all patients and, in curative patients, additional PV tumor-draining blood and postoperative blood (within 1 week after resection) were drawn. Additional follow-up blood samples (n = 38) could be obtained from 19 patients of the curative cohort during routine follow-up visits at our outpatient department up to 18 months after surgery.

BLOOD COLLECTION AND PROCESSING

In total, n = 221. 7.5 mL of EDTA blood samples were collected and processed within 2 hours after collection. Baseline peripheral blood was collected from all patients (n = 108). PV tumor-draining blood (n = 21) and post-operative peripheral blood samples (n = 54) were drawn from curative patients (Supplemental Fig. 1). Intraoperative draw via direct puncture was not possible for all patients due to challenging intraoperative anatomy (avoidance of potential bias due to tumor manipulation) or minimally invasive surgery.

Plasma was isolated by standard 2-step centrifugation protocol (10 min 300g and 10 min 1800g) from 7.5 mL EDTA blood and stored at −80°C until cfDNA extraction. CfDNA extraction was performed using the QiAmp Circulating Nucleic Acid Kit (Qiagen), using an elution volume of 25 µL. The cfDNA concentration was measured using the Qubit 4 Fluorometer (Thermo-Fisher Scientific) and stored at −20°C until ctDNA analysis.

KRAS CTDNA ANALYSIS

Analysis of mutant-KRAS status in cfDNA was performed using the ddPCR assays for the relevant hotspot mutations G12D, G12V, G12R, G12C, and Q61H (Bio-Rad Laboratories). Each sample was tested in triplicate for the same mutation present in tumor tissue. For samples where the mutant-KRAS status of the tumor could not be obtained (n = 24) or the patients had no KRAS mutation in tumor tissue (n = 23), a Bio-Rad G12/G13 Screening assay was used.

Then, 1 µL of ddPCR Supermix (no dUTP) (Bio-Rad Laboratories) was mixed with 1.1 µL of

Table 1. OS—curative and palliative study cohort and recurrence-free survival—curative study cohort.

(A) OS curative		n = 60[a]	OS curative cohort		
			Median OS, months (95% CI)	HR (95% CI)	P value
Age	≤67 years	29	22.0 (18.5–25.5)[b]	reference	0.614
	>67 years	31	21.2 (17.9–24.5)[b]	0.7 (0.2–2.6)	
Gender	male	28	20.2 (17.0–23.5)[b]	reference	0.385
	female	32	23.9 (17.0–23.5)[b]	0.6 (0.2–2.0)	
ECOG	0	31	21.2 (18.3–24.1)[b]	reference	0.982
	1	23	22.3 (18.2–26.3)[b]	1.0 (0.3–3.8)	
	2	6	20.5 (14.2–26.8)[b]	1.2 (0.1–10.6)	
UICC stage	I-II	45	24.8 (22.7–26.8)[b]	reference	**0.002**
	III	15	14.4 (10.1–18.7)[b]	5.8 (1.6–21.0)	
KRAS mutant copies[c]	<4	40	23.5 (20.8–26.3)[b]	reference	**<0.001**
	≥4	7	3.0 (0.4–5.6)	7.3 (1.9–28.0)	
R status	R0; CRM-	30	20.5 (17.4–23.6)[b]	reference	0.580
	CRM + /R1	30	23.5 (20.3–26.7)[b]	0.7 (0.2–2.5)	
Grading[d]	G2	37	22.5 (19.3–25.7)[b]	1.4 (0.4–5.0)	0.601
	G3	19	17.4 (14.2–20.5)[b]	reference	
Neoadjuvant treatment	no	48	20.4 (17.8–23.1)[b]	3.7 (0.5–30.2)	0.183
	yes	12	25.6 (23.1–28.1)[b]	reference	
Adjuvant treatment	no	5	6.0 (0.0–14.6)	5.9 (1.5–23.1)	**0.010**
	yes	55	22.1 (20.2–24.1)[b]	reference	
Clavien–Dindo	0–2	37	24.3 (21.8–26.8)[b]	reference	0.111
	3–4	23	20.0 (12.8–27.2)	2.7 (0.8–9.5)	
Ca 19-9	<500 U/mL	48	22.9 (20.2–25.6)[b]	reference	0.736
	≥500 U/mL	12	17.2 (20.3–25.1)[b]	1.3 (0.3–4.9)	

(B) OS palliative		n = 43	OS palliative cohort		
			Median OS, months (95% CI)	HR (95% CI)	P value
Age	≤67 years	22	17.0 (7.3–26.7)	reference	**0.016**
	>67 years	21	4.0 (2.8–5.2)	2.8 (1.1–6.7)	
Gender	male	28	10.0 (3.6–16.4)	reference	0.598
	female	15	10.0 (0.7–19.3)	1.3 (0.5–3.3)	
ECOG	0	20	17.0 (0–35.9)	reference	**0.020**
	1	15	10.0 (3.5–16.5)	2.5 (0.8–7.3)	
	2	8	2.0 (0–6.2)	4.5 (1.4–14.1)	
UICC stage	III	5	17.0 (0–36.2)	reference	0.455
	IV	38	10.0 (5.1–14.9)	1.7 (0.4–7.4)	
KRAS mutant copies[c]	<4	19	27.0 (1.9–52.1)	reference	**0.016**
	≥4	15	4.0 (3.1–16.9)	3.4 (1.1–9.9)	

(C) Multivariate OS curative			OS curative cohort	
			HR (95% CI)	P value
KRAS mutant copies	<4		reference	**0.008**

Continued

Table 1. (continued)				

(C) Multivariate OS curative		OS curative cohort		
		HR (95% CI)		P value
	≥4	6.9 (1.7–29.0)		
Adjuvant treatment	no	6.8 (1.5–30.0)		**0.013**
	yes	reference		

(D) Multivariate OS palliative		OS palliative cohort		
		HR (95% CI)		P value
KRAS mutant copies	<4	reference		**0.011**
	≥4	4.9 (1.4–17.0)		
ECOG	0	reference		**0.009**
	1	1.46 (0.4–5.3)		0.565
	2	11.5 (2.2–59.2)		**0.003**
Age	≤67 years	reference		**0.005**
	>67 years	6.0 (1.7–21.5)		

(E) Univariate analyses—RFS in the curative cohort

Univariate analyses		N = 60[a]	RFS curative cohort		
			Median RFS, month (95% CI)	HR (95% CI)	P value
Age	≤ 67 years	29	10.0 (8.6–11.5)	reference	0.097
	>67 years	31	11.0 (12.6–20.5)[b]	0.5 (0.2–1.2)	
Gender	male	28	11.0 (7.4–14.6)	0.9 (0.4–1.9)	0.712
	female	32	12.0 (6.3–17.7)	reference	
ECOG	0	31	9.0 (5.8–12.2)	reference	0.781
	1	23	12.0 (7.7–16.3)	0.8 (0.4–1.8)	
	2	6	15.5 (6.3–24.7)[b]	0.7 (0.2–3.0)	
UICC stage	I-II	45	16.0 (7.6–24.4)	reference	**<0.001**
	III	15	6.0 (1.0–11.0)	3.9 (1.7–8.7)	
R status	R0; CRM−	30	10.0 (5.9–14.1)	reference	0.081
	R0; CRM +/R1	30	16.0 (9.4–22.6)	0.5 (0.2–1.1)	
Grading[d]	G2	37	11.0 (6.2–15.8)	1.2 (0.5–2.8)	0.620
	G3	19	10.0 (6.3–13.7)	reference	
Neoadjuvant treatment	no	48	16.0 (9.9–22.1)	reference	**0.041**
	yes	12	10.0 (7.6–12.4)	2.2 (1.0–5.0)	
Adjuvant treatment	no	5	5.0 (0.0–11.4)	0.5 (0.2–1.3)	0.119
	yes	55	11.0 (8.6–13.4)	reference	
Clavien–Dindo	0–2	37	15.0 (8.6–21.4)	reference	0.510
	3–4	23	11.0 (9.4–12.6)	1.3 (0.6–2.8)	
Ca 19-9	<500 U/mL	48	11.0 (6.8–15.2)	0.9 (0.4–2.2)	0.881
	≥500 U/mL	12	11.0 (8.4–13.6)	reference	
KRAS mutant copies baseline[c]	<4	40	15.0 (9.2–20.8)	reference	**0.001**
	≥4	7	3.0 (0.4–5.6)	3.4 (1.2–9.7)	

Continued

Table 1. (continued)					
(E) Univariate analyses—RFS in the curative cohort					
			RFS curative cohort		
Univariate analyses		N = 60[a]	Median RFS, month (95% CI)	HR (95% CI)	*P* value
KRAS mutant copies Postoperative	<4	28	17.2 (13.5–20.9)[b]	reference	**0.014**
	≥4	9	3.0 (1.5–4.5)	3.4 (1.2–9.7)	
KRAS mutant copies portal venous	<4	15	19.3 (14.8–23.9)[b]	reference	**<0.001**
	≥4	3	3.0 (0–6.2)	4.6 (1.6–13.3)	
(F) Multivariate analyses for baseline blood					
			RFS		
Multivariate analyses			HR (95% CI)		*P* value
UICC stage	I-II		reference		**<0.001**
	III		6.0 (2.1–17.2)		
KRAS mutant copies Baseline	<4		reference		**0.033**
	≥4		3.7 (1.1–12.5)		
Adjuvant treatment	no		5.9 (1.7–20.7)		**0.006**
	yes		reference		
(G) Multivariate analyses for postoperative blood					
			RFS		
Multivariate analyses			HR (95% CI)		*P* value
UICC stage	I-II		reference		**0.004**
	III		7.4 (1.9–28.5)		
KRAS mutant copies Postoperative	<4		reference		**0.044**
	≥4		3.4 (1.0–10.9)		
Adjuvant treatment	no		6.7 (1.8–25.1)		**0.005**
	yes		reference		

(A + B + E) Univariate analyses (*P* values, log-rank test). (C + D + F + G) Multivariate analyses.
OS, overall survival; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; UICC, Union for International Cancer Control; CRM, circumferential resection margin; Ca 19-9, Carbohydrate Antigen 19-9.
Significant *P* values <0.05 are bolded.
[a]n = 5 perioperatively deceased patients not included in the analyses.
[b]Median not reached; mean was used.
[c]Mutant KRAS status after exclusion of perioperatively deceased patients: available for n = 47 curative patients and n = 34 palliative patients.
[d]Grading not available for n = 4 patients.

ddPCR Mutation detection assay (Bio-Rad Laboratories) and 10 units of HindIII Restriction enzyme (New England Biolabs) to create the Supermix. Next, 12.6 µL of the Supermix was mixed with up to 20 ng of the patient's cfDNA diluted to a volume of 9.4 µL. To avoid carryover of bubbles, 20 µL were transferred onto a QX100 droplet generator (Bio-Rad, Laboratories). After droplet generation, each well was carefully transferred into the ddPCR 96-well plate (Bio-Rad Laboratories), which was heat-sealed using the PX1 PCR Plate sealer (Bio-Rad Laboratories). The ddPCR 96-well plate was transferred to the Biometra T Professional Standard Gradient 96 PCR-Cycler (Analytik Jena) using the following settings: 95°C for 10 min, 40 cycles of (94°C for 30 s, 60°C for 60 s), and subsequently 98°C for 10 min.

The PCR plates were read by the QX100 Droplet Reader using Quantasoft software v.1.7.4 (Bio-Rad) to assess the number of droplets that were positive for wild-type and mutant-*KRAS* mutation. A sample was called positive for ctDNA if 3 or more droplets were positive for *KRAS* mutations. The average number of partitions measured was 13 254 (SD: 3201). The absolute number of copies per mL of plasma was calculated using the following equation:

$$\frac{\text{Copies}}{\text{mL plasma}} = \frac{\left(\frac{\text{copies}}{\mu L \text{ of reaction}} \times \text{reaction volume} \times \frac{cfDNA \text{ elution volume}}{input\ volume}\right)}{\text{volume of plasma used for } cfDNA \text{ extraction}}$$

(18, 19). In each PCR run, nontemplate controls, wild-type, and mutant-*KRAS* genomic DNA were included.

STUDY ENDPOINTS

The findings of the different time points and sites of blood draw were compared regarding *KRAS* ctDNA detectability (primary endpoint), mutation load, and correlation with OS and recurrence-free survival (RFS) (secondary endpoints).

STATISTICAL ANALYSIS

Statistical analyses were performed using the IBM SPSS v.27 (IBM Corporation) and GraphPad PRISM. The chi-square test (categorical data) and Fisher exact test (qualitative variables) were used for clinical correlation analyses. Kruskal–Wallis, followed by the Dunn multiple comparisons test, and the Wilcoxon matched-pairs signed-rank test was used for cfDNA and ctDNA detection and amount comparisons. The OS was computed as the period from the date of surgery or date of first diagnosis (palliative cohort or patients with neoadjuvant treatment) to either the date of death or last follow-up. The RFS was defined as the period from the date of surgery to the date of recurrence or tumor-related death, whichever occurred first. In-hospital mortality (Clavien—Dindo 5) led to exclusion from survival analysis (20). Survival curves for RFS and OS were plotted using the Kaplan–Meier method and analyzed using the log-rank test.

Both mutant-*KRAS* yes/no status and different mutant copy/mL plasma were analyzed for survival analyses. Based on the statistical tool "Cutoff finder" https://molpathoheidelberg.shinyapps.io/CutoffFinder_v1/ for the determination of cutoff points in molecular data the most statistically significant threshold was reached at a cutoff value of ≥ 4 mutant-*KRAS* copies/mL plasma—and was used for all survival analyses (21).

The Cox regression model (backward elimination [Wald]) was used for multivariate analysis to assess the prognostic value of positivity for mutant-*KRAS* ctDNA (Table 1). Results are presented as hazard ratio (HR) and 95% confidence interval (CI). Significant statements refer to P values of two-tailed tests that were <0.05.

Results

TUMOR TISSUE *KRAS* MUTATION STATUS

In our patient cohort, 84/108 tissue samples allowed for *KRAS* mutation analysis (for G12D, G12V, G12R, G12C, and Q61H). In 61/84 (72.6%) patients, the tumor showed a mutated *KRAS* status with 59.0% (n = 36) G12D, 24.6% (n = 15) G12V, 13.1% (n = 8) G12R, and 3.3% (n = 2) G12C. None of the patients had a Q61H mutation. No *KRAS* mutations were detected in 27.6% (n = 23).

CFDNA CONCENTRATIONS IN PDAC PATIENTS

The mean cfDNA concentration (corrected for plasma input volume) at baseline (n = 108) was 34.8 ng/mL (SD = 67.8) plasma. A significantly increased cfDNA concentration was detected in both portal vein and postoperative blood compared to baseline samples (both $P < 0.001$) (Fig. 1B). This was also seen in matched samples where a significantly higher cfDNA concentration was found in PV blood compared to baseline blood (22.23 ng/mL vs 55.12 ng/mL; $P < 0.0001$) (Fig. 1E).

PLASMA *KRAS* MUTATION STATUS

In total, 38.0% (41/108) of baseline plasma samples showed a *KRAS* mutation. The distribution of different mutations is shown in Fig. 1A. The matching mutation was assessed in the plasma for patients with known KRAS status. In 24 of the 61 (39.3%) patients with a *KRAS* mutation in tumor tissue, the corresponding mutation was found in the plasma. In patients with wild-type *KRAS* in their tumor tissue (n = 23), no *KRAS* mutations were detectable in cfDNA.

KRAS CTDNA MUTANT STATUS

At baseline, *KRAS* ctDNA was found in 38.0% of the whole cohort (n = 41/108). A significantly higher *KRAS* ctDNA detection rate was observed in palliative patients' plasma (n = 25/43, 58.1%) compared to curative patients' preoperative plasma (n = 16/65, 24.6%, $P < 0.01$) (Fig. 1C). In 23.8% of the intraoperative PV liquid biopsies, a *KRAS* mutation was detected (n = 5/21, 23.8%). In curative patients, *KRAS* mutations were also detected in 18.5% of peripheral blood after resection (n = 10/54).

For all positive baseline samples, the median mutant copies/mL plasma was 6–13 (range 0.85–5083.00). Mutant-*KRAS* copy numbers in the palliative cohort were significantly higher than at all time points and sites of blood draw in the curative cohort ($P < 0.0001$, Fig. 1D)—and for the matched curative patients, all *KRAS* positive portal vein samples had significantly higher
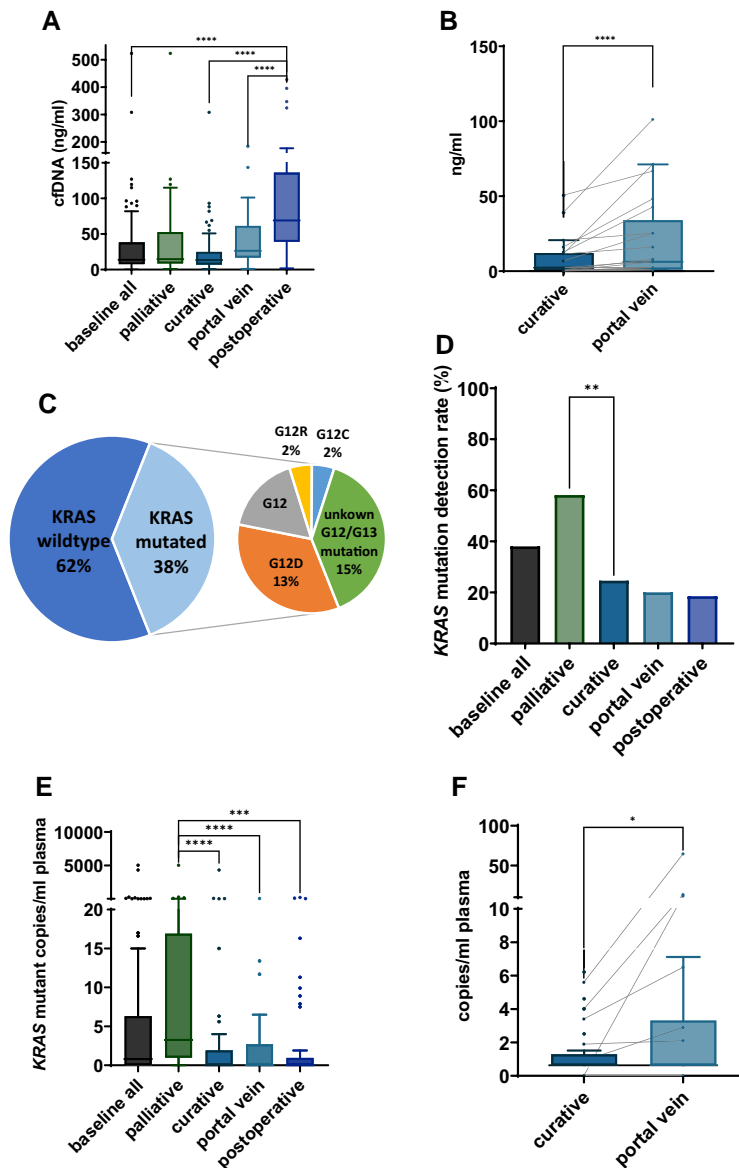
Fig. 1. Site dependent cfDNA and KRAS liquid biopsy analyses. (A), Chart of the distribution of KRAS mutations in baseline plasma (n = 105). KRAS status was assessed in cfDNA using the following ddPCR™ Mutation Detection Assays: G12D (dHsaMDV2510596), G12R (dHsaMDV2510590), G12V (dHsaMDV2510592), G12C (dHsaMDV2510584), Q61H (dHsaMDV2010131), and G12/G13 Screening Kit #1863506; (B), Mean cfDNA concentrations of all 105 analyzed patients; baseline blood from all, curative and palliative patients, and PV as well as postoperative plasma samples from curative patients; significant differences in cfDNA concentration at baseline and postoperative as well as PV and postoperative (****P < 0.0001); (C), ctDNA detection rate (all analyzed patients) of: baseline blood from all, baseline curative and palliative patients, and PV and postoperative plasma samples from curative patients; significant difference in detection rate in the palliative and curative cohort (**P < 0.01); (D), ctDNA copies per mL of plasma; significant difference between the palliative preoperative blood and all timepoints of the curative cohort (****P < 0.0001; ***P < 0.001); (E), The significant difference in cfDNA amounts between matched baseline (n = 21) and PV blood of curative patients (****P < 0.0001); (F), The significant difference in ctDNA copies per ml of plasma in matched pairs of baseline and PV blood (n = 6, *P < 0.05).

mutant copy numbers compared to the baseline sample with an average 6.4-fold increase ($P < 0.05$, Fig. 1F).

A significant correlation was found between KRAS status and grading ($P = 0.034$), with no correlation for the other histopathological parameters (including prognostic borderline resectability indicated by elevated Ca 19-9) in the curative study cohort ($n = 65$) (Table 2).

PROGNOSTIC RELEVANCE OF *KRAS* CTDNA MUTANT STATUS AT BASELINE

In both the palliative and curative cohort, patients with ≥4 mutant copies/mL plasma at baseline showed a significantly shorter OS ($P = 0.016$ and $P < 0.001$, respectively, Fig. 2A and B). For the curative cohort, a median OS of 3 months (95% CI: 0.4–5.6 months) survival was observed for those with ≥4 copies vs median OS not reached for those with <4 copies. The multivariate analysis revealed that ctDNA with ≥4 copies was an independent prognostic factor for shorter OS in both cohorts (palliative: HR 4.9 [range 1.4–17.0], $P = 0.011$ and curative: HR 6.9 [1.7–29.0], $P = 0.008$, Table 1).

RFS for baseline samples was performed for the cohort of curative patients with a KRAS mutant tumor. In the univariate analysis of RFS, the detection of ≥4 copies of *KRAS* ctDNA was significantly associated with shorter RFS (median 3 vs 15 months after resection) ($P = 0.001$) (Fig. 2C). The copy number cutoff of ≥4 copies in the curative patient cohort (HR 3.7 [range 1.1–12.5]; $P = 0.033$) was an independent prognostic marker in the multivariate analysis (Table 1).

PROGNOSTIC RELEVANCE OF *KRAS* CTDNA MUTANT STATUS FOR PORTAL VENOUS AND POSTOPERATIVE BLOOD

We collected PV blood from 21 curative patients, out of whom 19 patients had a mutant-KRAS status. For the RFS univariate analysis, the detection of ≥4 copies KRAS ctDNA in the portal vein was significantly associated with shorter RFS (median 3 months after resection vs median survival not reached) ($P < 0.001$) (Fig. 2D). The low number of patients did not allow multivariate analyses for this cohort. In addition, postoperative peripheral blood was analyzed in 41 curative patients with mutant-KRAS status out of 54 collected patients. For the RFS univariate analysis, the detection of ≥4 copies were significantly associated with shorter RFS (median 3 vs 17 months after resection) ($P = 0.014$) (Fig. 2E). The detection of the postoperative copy number cutoff of ≥4 copies was an independent prognostic marker in the multivariate analysis (HR 3.4, range 1.0–10.9; $P = 0.044$, Table 1).

KRAS CTDNA AS A FOLLOW-UP MARKER IN RESECTED PATIENTS

Follow-up samples were obtained in 19 resected patients. Out of these, 38 follow-up blood samples were analyzed during a follow-up time of up to 18 months. Thirteen events of tumor recurrence were observed. A high correlation between the occurrence of relapse and *KRAS* ctDNA positivity was observed (Fig. 3). Among the relapsed patients, 11 showed increased levels of *KRAS* mutant copies, of which KRAS detection proceeded the clinical manifestation of relapse in 3 patients. Interestingly, Ca 19-9 levels were not >500 U/mL in 5/11 patients with relapse, but 4 were *KRAS* positive. Patient 072 was negative during the first 4 time points. Then 9 months after operation, 0.5 mutant copies/mL were detected. At this point, Ca 19-9 was 377 U/mL, and no sign of relapse in the computed tomography scan was observed. After 3 months, the patient had >1200 mutant copies, a Ca 19-9 level of >78 000 U/mL, and metastases were detected in the liver. The metastases grew rapidly, and the patient died 2 months after the relapse. Also, in patients 134 and 73, *KRAS* ctDNA analyses indicated a relapse 3 months before the clinical manifestation. In patient 134, the preoperative blood showed a high mutation load with 52 mutant copies/mL (Ca 19-9: 3 155 U/mL), 6 months after operation 0.6 mutant copies/mL was detected (Ca 19-9: 152 U/mL), and at 9 months, 6.1 mutant copies (Ca 19-9: 3 155 U/mL) were detected. Three months later, the patient was diagnosed with liver metastases. Patient 73 had 3.4 mutant copies/mL (Ca 19-9: 12 U/mL) before surgery. After surgery, no mutant copies could be detected, but after 3 months, again 2.2 mutant copies/mL were detected. After 6 months, the patient still had a detectable mutation load of 0.8 mutant copies/mL (Ca 19-9: 24 U/mL), and the patient developed liver metastasis. Interestingly, Ca 19-9 levels never exceeded 60 U/mL at any time point, whereas ctDNA was detected early on. In contrast, in patient 001, *KRAS* ctDNA was at all time points analyzed negative, and the patient is still negative after 24 months RFS—indicating complete remission at this moment.

Discussion

This study demonstrated that irrespective of the site of blood draw or time point, mutant-*KRAS* detection in the blood of PDAC patients is significantly associated with dismal prognosis for palliative and curative patients. In curative patients, mutant-*KRAS* detection might serve as an effective tool for identifying prognostic borderline patients who should receive neoadjuvant treatment despite the anatomical resectability of the primary tumor.

To our knowledge, this study is the first to evaluate the value of tumor-draining PV blood for ctDNA-based liquid biopsy in PDAC. Furthermore, the detection of *KRAS* mutations (with ≥4 copies/mL) in PV blood was associated with an extremely poor prognosis, while at the same time, the mutation load was significantly

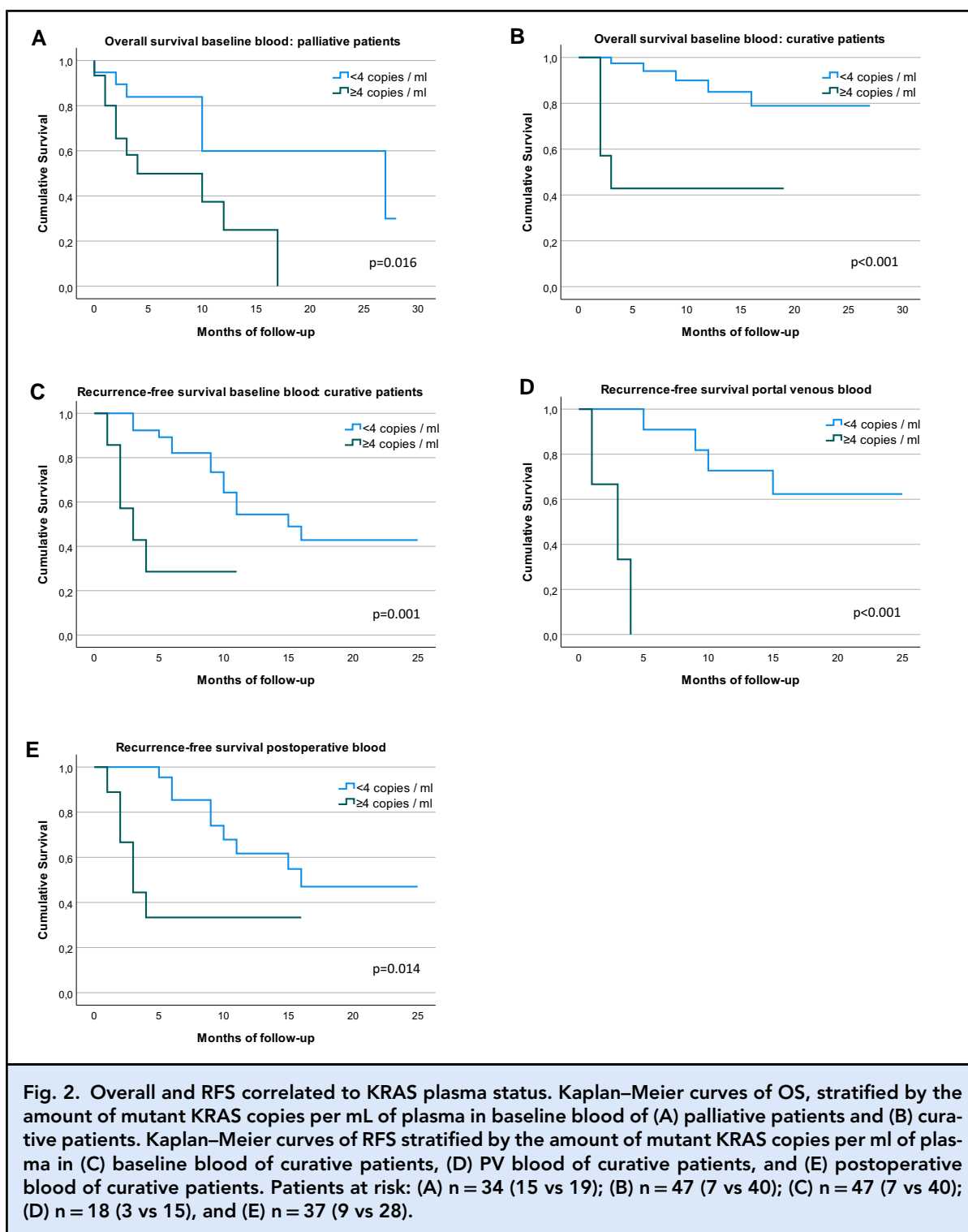| Table 2. Clinical characteristics and plasma KRAS status of the curative cohort.								
				Plasma KRAS mutant status (n = 51)				
		Curative patients (n = 65)		baseline KRAS negative n = 35		baseline KRAS positive n = 16		
		n	%	n	%	n	%	P value
Age	≤67 years	30	46.2	16	45.7	8	50.0	1.000
	>67 years	35	53.8	19	54.3	8	50.0	
Gender	male	31	47.7	17	48.6	8	50.0	0.749
	female	34	52.3	18	51.4	8	50.0	
ECOG	0	32	49.2	19	54.3	6	37.5	0.330
	1	26	40.0	12	34.3	9	56.3	
	2	7	10.8	4	11.4	1	6.2	
Neoadjuvant treatment	yes	12	18.5	3	8.6	3	18.8	0.363
	no	53	81.5	32	91.4	13	81.2	
Surgical procedure	PD/PPPD	41	63.1	26	74.3	8	50.0	0.177
	left pancreatectomy	8	12.3	2	5.7	3	18.8	
	total pancreatectomy	16	24.6	7	20.0	5	31.2	
Adjuvant treatment	yes	55	84.6	31	88.6	12	75.0	0.240
	no[a]	10	15.4	4	11.4	4	25.0	
Dindo classification	0–2	37	56.9	18	51.4	10	62.5	
	3–4	23	35.4	15	42.9	4	25.0	
	5	5	7.7	2	5.7	2	12.5	0.401
pT stage	T1–2	37	56.9	22	62.9	9	56.3	0.654
	T3–4	28	43.1	13	37.1	7	43.7	
pN stage	N0	18	27.7	11	31.4	4	25.0	0.640
	N + (N1/2)	47	72.3	24	68.6	12	75.0	
Grading[b]	G2	41	67.2	26	74.3	7	43.8	**0.034**
	G3	20	32.8	9	25.7	9	56.2	
R status	R0, CRM-	32	49.2	17	48.6	8	50.0	0.925
	R0, CRM +/R1	33	50.8	18	51.4	8	50.0	
UICC	I-II	49	75.4	29	82.9	11	68.8	0.256
	III	16	24.6	6	17.1	5	31.2	
Ca 19-9	≤500 U/mL	48	73.8	25	71.4	11	68.8	0.846
	>500 U/mL	17	26.2	10	28.6	5	31.2	
Recurrence	yes	29	44.6	14	40.0	7	43.8	1.000
	no	36	55.4	21	60.0	9	56.2	

ECOG, Eastern Cooperative Oncology Group; CRM, circumferential resection margin; Ca 19-9, Carbohydrate Antigen 19–9; UICC, Union for International Cancer Control; PD, partial pancreatoduodenectomy; PPPD, pylorus preserving.
Significant P values <0.05 are bolded.
[a]Not started during follow-up period, or due to reduced ECOG or death.
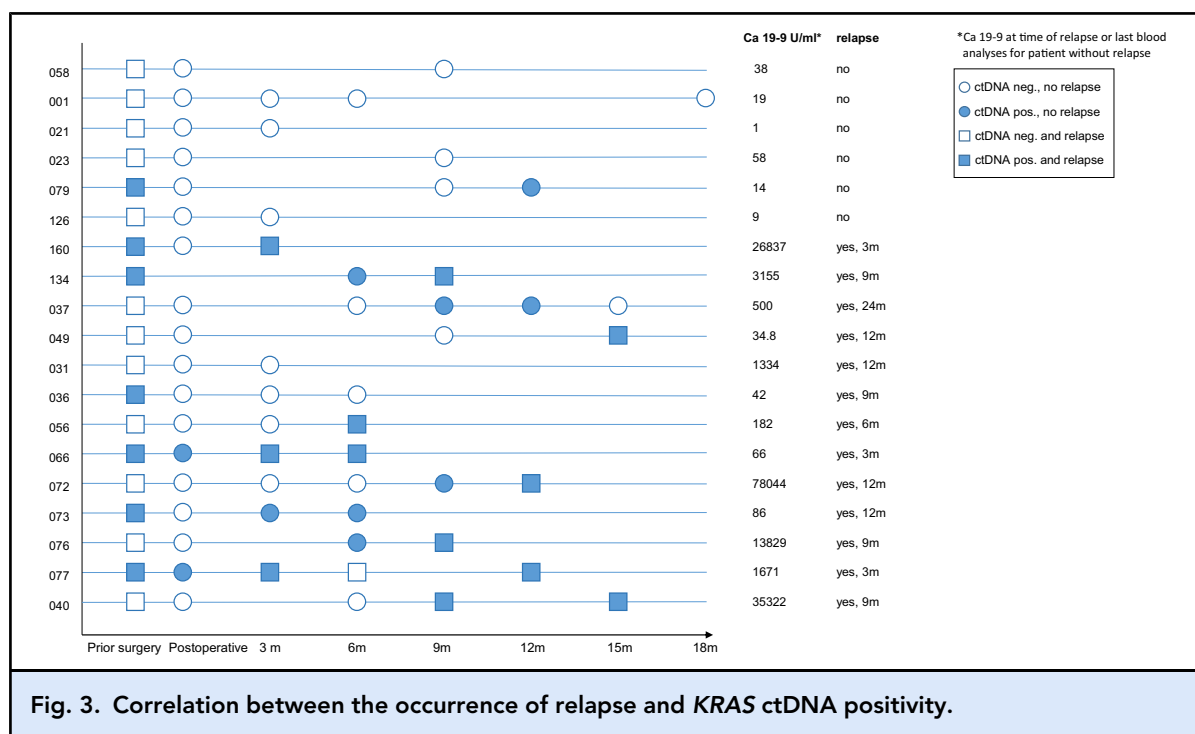[b]For n = 4 patients no grading (G) is available.

Fig. 2. Overall and RFS correlated to KRAS plasma status. Kaplan–Meier curves of OS, stratified by the amount of mutant KRAS copies per mL of plasma in baseline blood of (A) palliative patients and (B) curative patients. Kaplan–Meier curves of RFS stratified by the amount of mutant KRAS copies per ml of plasma in (C) baseline blood of curative patients, (D) PV blood of curative patients, and (E) postoperative blood of curative patients. Patients at risk: (A) n = 34 (15 vs 19); (B) n = 47 (7 vs 40); (C) n = 47 (7 vs 40); (D) n = 18 (3 vs 15), and (E) n = 37 (9 vs 28).

Fig. 3. Correlation between the occurrence of relapse and *KRAS* ctDNA positivity.

higher. Positive patients had a median RFS of only 3 months compared to median RFS not reached in ctDNA negative patients, showing the highest predictive power of all blood-draw sites.

In curative patients, our results demonstrate that detection of *KRAS* ctDNA is a strong independent prognostic biomarker for poor RFS when detected in pre- and postoperative blood. *KRAS* status did not correlate with Ca 19-9 levels, indicating an independent role of these 2 biomarkers.

In general, *KRAS* mutant ctDNA is detectable preoperatively in 24.6% of patients with curative disease, which is consistent with other studies set in the preoperative stage (2–28). In palliative patients, the baseline ctDNA detection rate of 73.5% was significantly higher, in line with former studies on ctDNA in advanced stage PDAC (29–31).

The detected increases in postoperative cfDNA compared to baseline can be attributed to the massive cfDNA release after major surgical intervention. At the same time, the resection of the primary tumor did not lead to a significant drop in postoperative ctDNA positivity rate, indicating the presence of residual occult disease in many curative patients.

Our follow-up data indicated that *KRAS* ctDNA also serves as a good follow-up marker for recurrent disease detection and might be useful alongside the current standard diagnostics, including Ca 19-9 and computed tomography.

Even though we performed a prospective study, shortcomings are the short follow-up period, lack of a marker for patients with no KRAS mutation, and—as the main limitation—the relatively small sample size, which causes a lack of statistical power for subgroup analysis and the multivariate model, reducing robustness and reproductivity of results. This may also affect, e.g., the nonsignificant impact of Ca 19-9 levels within our study cohort. As PV blood and postoperative blood were not always obtained (considering the patients' intra- and postoperative conditions), a comparison of matched blood samples was not possible for all patients.

Still, our explorative study results are highly significant, justify this approach, and are supported by recent data on lung cancer where early postoperative ctDNA was associated with dismal prognosis (32).

In addition, this is the first ctDNA-based study where samples are collected intraoperatively directly from tumor-draining vessels with significant results despite the low number of patients. The presented data are necessary before attempting a EUS-guided puncture of the portal vein, which might have a genuine potential significant impact on individual treatment strategies.

However, multicenter prospective clinical studies are required to further evaluate the clinical impact of different time points and blood-draw sites for prognosis estimation in PDAC patients and thus as an additional tool for reliable identification of prognostic borderline patients. Furthermore, additional research in studies on extracellular mutant *KRAS* might provide valuable technical improvements of this diagnostic approach. Lately, studies have indicated extracellular vesicles as

an important source of mutant-*KRAS* DNA in plasma from PDAC patients (33–35).

In conclusion, our study confirms an independent prognostic value of ctDNA detection in PDAC patients. For the first time, it was shown that liquid biopsies from the portal vein might have the lowest risk of false-negative results due to the higher mutation load compared to other sites and might therefore be the most crucial side for reliable prediction of short RFS in clinical routine. The clinical translation of portal vein liquid biopsies is safely possible by endosonographic ultrasound-guided puncture of the portal vein in combination with the fine-needle aspiration of the primary tumor, which is standard procedure in nonmetastatic patients (36). The presented results form the basis for future studies evaluating the endosonographic ultrasound-guided puncture of the portal vein for liquid biopsies, as PV liquid biopsies for ctDNA analysis might serve as an additional biomarker for identifying prognostic borderline patients who should receive neoadjuvant treatment despite anatomical resectability of the primary tumor.

Supplementary Material

Supplementary material is available at *Clinical Chemistry* online.

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