Longitudinal Analysis of Cell-free Mutated KRAS and CA 19-9 Predicts Survival Following Curative Resection of Pancreatic Cancer

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Abstract

Background: Novel biomarkers and molecular monitoring tools hold potential to improve outcome for patients following resection of pancreatic ductal adenocarcinoma (PDAC). We hypothesized that the combined longitudinal analysis of mutated cell-free plasma *KRAS* (cf*KRAS*^{mut}) and CA19-9 during adjuvant treatment and follow-up might more accurately predict disease course than hitherto available parameters.

Methods: Between 07/2015 and 10/2018, we collected 134 plasma samples from 25 patients (pts) after R0/R1-resection of PDAC during adjuvant chemotherapy and post-treatment surveillance at our institution. Highly sensitive discriminatory multi-target ddPCR assays were employed to screen plasma samples for cf*KRAS*^{mut}. cf*KRAS*^{mut} and CA19-9 dynamics were correlated with recurrence-free survival (RFS) and overall survival (OS). Patients were followed-up until 01/2020.

Results: Out of 25 enrolled patients, 76% had undergone R0 resection and 48% of resected PDACs were pN0. 17/25 (68%) of patients underwent adjuvant chemotherapy. Median follow-up was 22.0 months, with 19 out of 25 (76%) pts relapsing during study period. Median RFS was 10.0 months, median OS was 22.0 months. Out of clinicopathologic variables, only postoperative CA19-9 levels and administration of adjuvant chemotherapy correlated with survival endpoints. cf*KRAS*$^{mut.}$ was detected in 12/25 (48%) of patients, and detection of high levels inversely correlated with survival endpoint. Integration of cf*KRAS*^{mut} and CA 19-9 levels outperformed either individual marker. cf*KRAS*^{mut} outperformed CA19-9 as dynamic marker since increase during adjuvant chemotherapy and follow-up was highly predictive of early relapse and poor OS.

Conclusions: Integrated analysis of cf*KRAS*^{mut} and CA19-9 levels is a promising approach for molecular monitoring of patients following resection of PDAC. Larger prospective studies are needed to further develop this approach and dissect each marker`s specific potential.

Background

Despite significant progress in understanding tumor genetics and the molecular mechanisms driving tumor development and resistance to therapy, only minor improvements have been achieved to date in the treatment of patients with pancreatic ductal adenocarcinoma (PDAC). With an average 5-year overall survival (OS) rate of only 10% across all stages, most patients still succumb to their disease, making PDAC one of the most aggressive tumor entities [1] [2] [3]. The only potentially curative treatment is surgical resection of early-stage tumors [4] [5]. However, recurrence rates even after R0 resection remain unacceptably high [6] [7] [8] [9] [10]. The integration of more efficacious systemic chemotherapy regimens has improved median overall survival [11], yet responses of individual PDACs to chemotherapy are highly heterogeneous and personalization of perioperative therapy is in its infancy [12] [13] [14] [15] [16] [17] [18] .

Consequently, the development and validation of novel biomarkers and molecular monitoring tools to predict disease course and assess efficacy of adjuvant chemotherapy are urgently needed. The analysis of tumor-derived cell-free nucleic acids (ctDNA) extracted from the plasma and other body fluids is a promising tool for molecular diagnostics and non-invasive monitoring of cancer patients [19] [20] [21] [22] [23] [24] [25]. Up to 95% of PDACs harbor activating hot spot mutations in *KRAS* which are readily detectable in the circulation of PDAC patients [20] [26] [27] [28]. We recently described the development and validation of highly sensitive single-target and discriminatory multi-target *KRAS* ddPCR assays for the analysis of cfDNA [29]. These assays allow identification and quantification of mutated *KRAS* directly from circulation without previous knowledge of tumor *KRAS* mutational status, which is not routinely tested for resectable PDACs.

For this study, we hypothesized that longitudinal assessment of cf*KRAS*^{mut} following curative resection of PDAC in combination with established protein biomarkers might better identify pts at risk for imminent tumor relapse, indicate failure of adjuvant treatment and ultimately guide treatment according to molecular monitoring. To study the feasibility of this approach, we analyzed plasma samples collected from patients undergoing adjuvant chemotherapy and post-treatment surveillance at our institution in a single-center retrospective biomarker study aiming to identify associations between cf*KRAS*^{mut} and CA19-9 dynamics and clinical outcome post PDAC resection.

Methods

Study design and population

25 patients were included in a retrospective observational single center biomarker study conducted at Freiburg University Medical Center. Local institutional review board (IRB) approved all study procedures (EK48/18). All patients provided written informed consent for sample collection and analysis. All patients underwent adjuvant chemotherapy and/or post-treatment surveillance at our institution. Inclusion criteria included status post R0 or R1 resection of pancreatic adenocarcinoma with curative intent within 8 weeks of first sample collection and availability of plasma samples for cfDNA extraction. Key exclusion criteria included R2 resection, evidence of metastatic disease on pre- or postoperative CT staging, histologies other than adenocarcinoma. Primary endpoint was detection of cf*KRAS*^{mut} in at least one sample during study period. Secondary endpoints included association between changes in cfKRAS^{mut} and relapse-free survival (RFS) and overall survival (OS). Additionally, clinical, pathologic, treatment- and outcome-related data were analyzed.

Extraction of cell-free DNA (cfDNA) from plasma samples

Blood samples were collected using commercially available EDTA tubes and plasma was extracted and frozen within one hour of collection. Plasma was extracted through two subsequent centrifugation steps at 3000 rpm and 14000 rpm, each for 10 min at 4 °C. Obtained plasma was stored at -80 °C until extraction of cfDNA. cfDNA was extracted from 4 ml plasma following the SEP/SBS protocol of the PME-

free circulating DNA extraction kit (Analytik Jena, cat. no. 845-IR-0003050), following manufacturer's instructions. Two subsequent elution steps with each 30 µl Elution Buffer were performed to optimize the yield of extracted cfDNA. DNA was stored at -20 °C until cfDNA quantification. cfDNA was evaluated with fragment analyzer and quantified using Qubit 2.0 fluorometer. In patients with resectable PDAC, DNA yield from 4 ml of plasma typically ranged from 1 to 20 ng/µl.

Droplet digital PCR (ddPCR)

ddPCR for cf*KRAS*^{mut} was performed as recently described (Hussung et al, 2020). In brief, cfDNA was screened for the presence of the 11 most commonly found *KRAS* hot spot mutations, in PDAC, covering more than 90% of PDAC cases. Highly sensitive single-target assays were used to confirm presence of the mutation identified. Primers and probes were manufactured by Integrated DNA Technologies (IDT, Inc., Coralville, Iowa, USA), sequences and all assay procedures are detailled in Hussung et al [29].

Limit of detection (LOD) and limit of blank (LOB) of the individual assays have been previously described [29] .

Statistical analysis

Recurrence-free survival (RFS) was defined as time from resection of PDAC to the first radiologic recurrence (local or distant) or death due to PDAC. Overall survival (OS) was defined as time from the date of diagnosis until death due to any cause. The Kaplan–Meier survival analysis was performed to calculate both RFS and OS. Univariate analyses were performed using the log-rank test. In order to explore independent prognostic factors for RFS and OS, we used backward stepwise Cox regression modeling to estimate hazard ratio (HR) with 95% confidence interval (CI). To compare independent variables, Chi-squared or Fisher's exact test and the Mann–Whitney (rank-sum) test were performed. All statistical analyses were performed using GraphPad Prism Version 5.03 (GraphPad Software, Inc., La Jolla, California, USA) and SPSS 25 software Version 1.0.0.1327 (IBM Corporation, New York, United States). *P* values < 0.05 were considered as statistically significant.

Results

Patient cohort

25 patients with nonmetastatic, R0/R1-resected adenocarcinoma of the pancreas were included in the study. Patient characteristics are summarized in Table 1. R0 resection rate was 76% (19/25),12/25 (48%) of tumors were nodal negative (pN0). 17/25 (68%) patients underwent adjuvant chemotherapy. Median follow-up for the cohort was 22.0 months, with 19 out of 25 (76%) pts relapsing during this period. Median RFS for the cohort was 10.0 months, median OS was 22.0 months. We performed univariate and multivariate survival analyses (Supplemental Tables 1 and 2, Figure S1) for established clinicopathologic variables and found a trend towards inferior RFS but not OS for R1 resection (Figure S1 A,B), a significant inverse correlation between elevated CA19-9 in the first sample collected after resection and RFS and OS

(Figure S1 C,D) and significantly better OS for patients undergoing adjuvant chemotherapy (Figure S1 E,F).

Table 1
Patient and tumor characteristics

Clinicopathologic features	n =25 (%)
Median age (years)	75
Age range	42 – 81
Sex	
Male	18 (72)
Female	7 (28)
Tumor location	
Pancreas head	20 (80)
Pancreas body & tail	5 (20)
T stage	
T1 – T2	7 (28)
T3	18 (72)
N status	
N0	12 (48)
N1-2	13 (52)
R status	
R0	19 (76)
R1	5 (20)
Rx	1 (4)
Lymphovascular invasion	
L0	15 (60)
L1	10 (40)
Perineural invasion	
Pn0	2 (8)
Pn1	23 (92)
Grading	
G2	13 (52)
G3	12 (48)

Adjuvant chemotherapy		
Yes	**1**	7 (68)
No		8 (32)
Time to relapse (months)		
Median		10
Range		0.5 – 42
Overall survival (months)		
Median		22
Range		0.5 - 46

Analysis of plasma cfKRAS^{mut}

We analyzed 134 plasma samples collected from 25 patients at routine follow-ups before, during and after adjuvant chemotherapy. First samples were taken at a median of 40 days (95% CI 26-50) after resection prior to adjuvant chemotherapy. Median number of samples collected was 4 samples per patient (95% CI 3-5 samples). Median time interval between sampling was 70 days (95% CI 63-91). We screened cfDNA extracted from plasma samples for the presence of cfKRAS^{mut} with recently described discriminatory multi-target *KRAS* ddPCR assays, covering the 11 most common *KRAS* hot spot mutations in PDAC [29]. At the postoperative stage, no molecular pathology data was available for any tumor. However, for a subset of patients *KRAS* mutational status became available at relapse (Supplemental Table 3).

Across all samples analyzed, cf*KRAS*^{mut} was detected in 34/134 (25%) samples and 12/25 (48%) of patients for at least one time point. In 16/16 (100%) patients with later on determined tumor tissue *KRAS* mutational status, the SNV detected by ddPCR in plasma (cf*KRAS*^{mut}) matched the *KRAS* SNV detected in tissue analysis (Supplemental Table 3), confirming the validity of ddPCR cf*KRAS*^{mut} analysis. In 0/134 (0%) plasma samples more than one *KRAS* SNV could be detected above assay threshold.

Association of cfKRAS^{mut} and elevated CA19-9 levels with survival endpoints

Detection of cf*KRAS*^{mut} at any time point during study course above assay threshold was not associated with RFS or OS (Figure 1 A, B). However, when a threshold of 15 copies *KRAS*^{mut} per ml plasma for cf*KRAS*^{mut} was chosen, *cfKRAS*^{mut} positivity at any time point during study period was strongly associated with early relapse and poor survival (Figure 1 C, D). CA19-9 levels were determined from the

same blood collections as part of clinical assessment. 12/25 (48%) of patients had at least one blood sample with CA19-9 above normal range during study course. Increased CA19-9 at any time point was associated with significantly inferior RFS and a trend towards inferior OS (Figure 1 E, F). Notably, only 6/12 (50%) patients were double positive for cf*KRAS*^{mut} and CA19-9, indicating that cf*KRAS*^{mut} and CA19-9 positivity are not redundant. Patients with either CA19-9 positivity or cf*KRAS*^{mut} levels > 15 copies/mL cfKRAS during study course (14/25, 56%) showed inferior RFS and OS, indicating that the integration of both biomarkers might be predictive and prognostic for a larger group of patients than assaying them individually (Figure 1 G, H). Survival of double positive patients was similar to single positive patients in our cohort (data not shown).

Association of cfKRAS^{mut} and CA19-9 dynamics with survival

Protein tumor markers and cfDNA are highly dynamic biomarkers for the molecular monitoring of disease course and treatment response. We therefore next analyzed whether changes over time in either biomarker are associated with outcome in our cohort. For each 9/18 (50%) patients with a sufficient number of follow-up samples, cf*KRAS*^{mut} or CA19-9 levels increased during observation period. Increase of cf*KRAS*^{mut} was associated with significantly reduced OS (Figure 2 A), while increase of CA19-9 was associated with a non-significant trend towards inferior OS (Figure 2 B). Similarly, early increase of cf*KRAS*^{mut}, defined as increase within 6 months after surgery, was strongly associated with inferior OS while early CA19-9 increase was associated with only a trend towards shorter OS (Figure 2 C, D). Integrating both markers for the analysis of dynamic changes over time did not outperform cf*KRAS*^{mut} alone (Figure 2), suggesting that cf*KRAS*^{mut} might be the biomarker of choice for longitudinal monitoring in this setting.

Single patient analysis

Figure 3 illustrates the relationship between cf*KRAS*^{mut} and CA19-9 (Figure 3A) dynamics and tumor relapse for individual patients. 13/18 patients in the analysis relapsed during observation period. Increase of cf*KRAS*^{mut} or CA19-9 was significantly associated with relapse. Single-patient analysis also illustrates dynamic changes during adjuvant chemotherapy and follow-up with several patients showing transient increases followed by decreases of either marker.

Figure 3B illustrates that in most patients, relapsed was proceeded by a strong increase of CA19-9 or cf*KRAS*^{mut}. However, single patient analyses also illustrate that both cf*KRAS*^{mut} and CA19-9 are both highly dynamic markers and that individual patterns are highly heterogenous. Larger cohorts and prospective trials are required to better unravel the relationships and the temporal relationship between biomarker dynamics and clinical relapse and to study the impact of therapeutic intervention.

Discussion

In an exploratory analysis, we followed a small cohort of pancreatic cancer patients after curative resection of pancreatic adenocarcinoma through adjuvant therapy and post-treatment follow-up. We analyzed mutated *KRAS* in cell-free DNA with discriminatory ddPCR assays and integrated results with CA19-9 levels for association with relapse and survival endpoints. Numerous studies have unveiled the potential of the analysis of cell-free mutated tumor DNA as novel diagnostic [26], predictive [30] [31] [32] and prognostic [30] [31] [33] [34] [35] [36] biomarker for pancreatic cancer.

What takes our study apart is the use of discriminatory multi-target *KRAS* ddPCR assays [29] to directly identify *KRAS* SNVs without performing previous tumor NGS. These assays have higher sensitivity compared to many available NGS-based assays (Hussung et al). In comparison to more sophisticated NGS panels specifically developed for cfDNA analysis [28] [37], multi-target ddPCR assays are associated with much lower assay costs, allowing for the serial analysis through clinical course analogous to CA19-9 levels. Using these assays, our cf*KRAS*^{mut} detection rate in the cohort was similar to other published data for patients following PDAC resection [35] [30] [20]. A 100% concordance rate between tumor tissue und detected cfDNA *KRAS* SNVs further validates our approach.

In our cohort, detection of cf*KRAS*^{mut} in the first postoperative sample alone did not significantly correlate with survival (data not shown), while elevated CA19-9 levels at first presentation were associated with poor outcome. Similarly, positivity for cf*KRAS*^{mut} at any time point above assay threshold alone was not significantly associated with survival. However, when choosing a more stringent cf*KRAS*^{mut} cut-off or when analyzing dynamic changes (increase vs non-increase), cf*KRAS*^{mut} was strongly associated with survival and outperformed CA19-9 levels for association with relapse and OS, highlighting the importance of identifying clinically validated cut-offs for cfDNA analysis [38] [39] [40] and also underlining the limitations associated with analyzing a small patient cohort.

One main finding of our analysis was that cf*KRAS*^{mut} positivity and CA19-9 elevation are only partially overlapping and that combining both parameters identifies a larger cohort of patient with poor outcome. Several studies have suggested integration of established and experimental protein biomarkers with cfDNA analysis for pancreatic cancer early diagnostics [27] [26] [41] [42] [43], identification of minimal residual disease [44] and molecular monitoring for advanced disease [40]. Our approach is focused on clinical applicability and feasibility through integration of the two relatively easy-to-assess biomarkers cf*KRAS*^{mut} analysis and CA19-9. And while our data clearly show that CA19-9 and cf*KRAS*^{mut} levels each have their own distinct advantages and disadvantages and that integrating them for analysis might be superior to analysing them individually, the question of how best to integrate both biomarkers for clinical practice remains challenging, which is also illustrated by the analysis of single patient`s disease course in our cohort.

Finally, much larger trials will be required to study the potential on biomarker-based therapeutic intervention for pancreatic cancer. Systemic treatment options for PDAC are limited to a small number of

combination chemotherapy regimens (PRODIGE 11, NEJM 2011, MPCAT trails NEJM 2013) and some recent developments in personalized treatment based on molecular profiling (POLO trial NEJM 2019). A switch of adjuvant chemotherapy regimen based on molecular monitoring appears feasible yet need extensive clinical validation in interventional trials, especially since established adjuvant treatment standards took so many years to establish.

In summary, our study proposed a clinically feasible way to assay cf*KRAS*^{mut} together with CA19-9 in patients following curative resection of PDAC. Through combination of both markers, patients could be better stratified in terms of relapse risk and overall prognosis.

List Of Abbreviations

cfDNA Cell-free DNA

cf*KRAS*^{mut} Mutated cell-free plasma *KRAS*

ctDNA Tumor-derived cell-free nucleic acids

ddPCR Droplet digital PCR

IDT Integrated DNA Technologies

IRB Institutional review board

HR Hazard ratio

LOB Limit of blank

LOD Limit of detection

mFOLFIRINOX 5-fluorouracil, irinotecan, oxaliplatin, leucovorin

NGS Next generation sequencing

OS Overall survival

PCR Polymerase chain reaction

PDAC Pancreatic ductal adenocarcinoma

pts Patients

RFS Recurrence-free survival

SNV Single nucleotide variants

Declarations

Ethics approval and consent to participate

Local institutional review board (IRB) approved all study procedures (EK48/18). All patients provided written informed consent for sample collection and analysis.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SH collected, analyzed and interpreted the patient data. SH, DA and RF were major contributors in writing the manuscript. SH, MB, UW and RF conceptualized the study. RF supervised the data collection, analysis and writing of the manuscript. JH, MF, RK, UP, FS, NB and JD contributed to data collection and manuscript edition. All authors read and approved the final manuscript.

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Figures

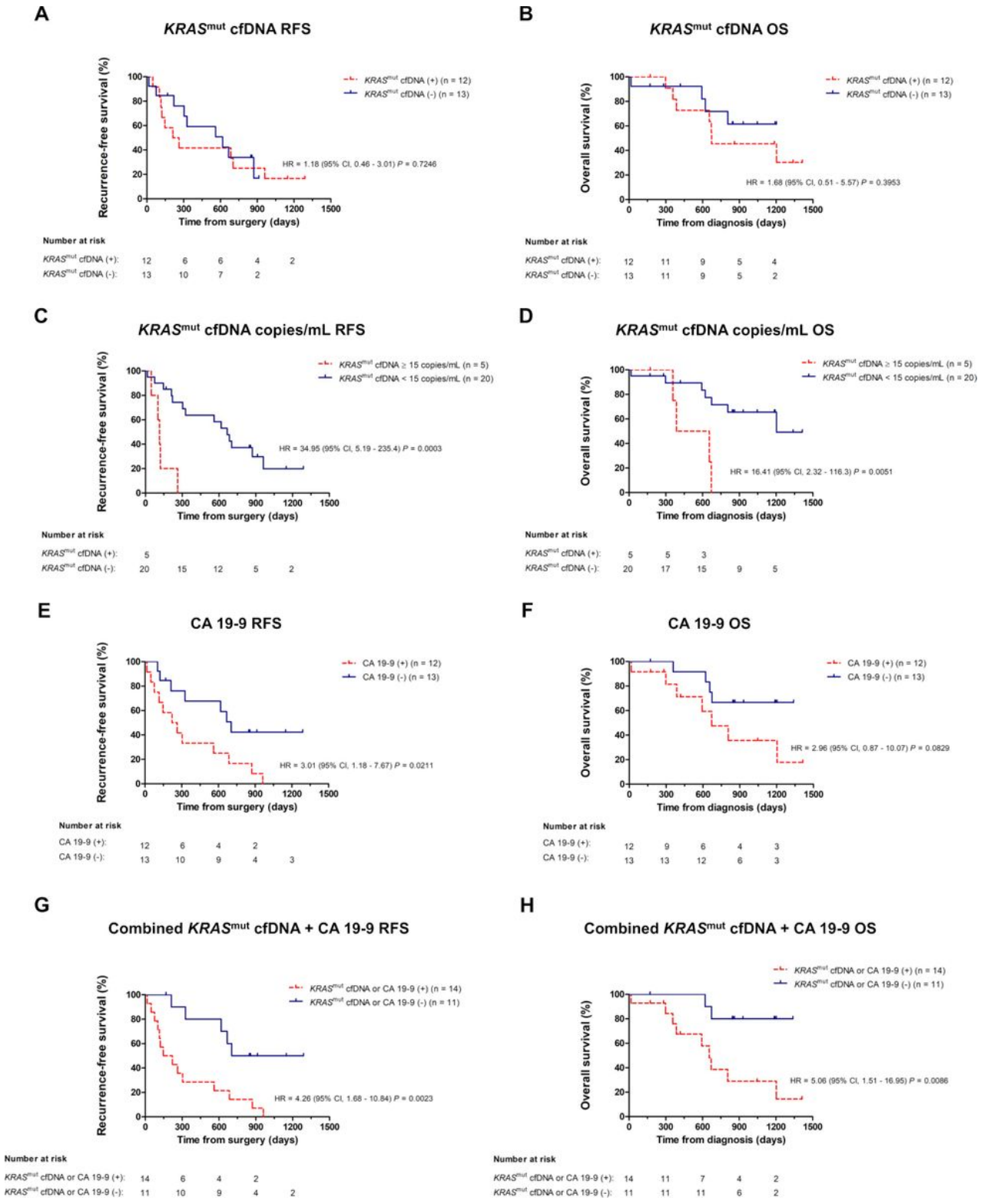

Figure 1

Association of cfKRASmut detection and elevated CA19-9 levels with survival endpoints (A, B) Kaplan-Meier estimates of RFS (A) and OS (B) for patients following curative resection of PDAC with versus without detectable cfKRASmut at any time point during study period. (C, D). A more stringent cfKRASmut cut-off level of > 15 copies/mL plasma was chosen. (E, F) Kaplan-Meier estimates of RFS (E) and OS (F) for resected PDAC patients with elevated (> 36 U/mL) versus normal (≦ 36 U/mL) CA19-9 levels at any

time point during observation period. (G, H) Kaplan-Meier estimates of RFS (G) and OS (H) for resected PDAC patients with either CA19-9 positivity or cfKRASmut levels > 15 copies/mL cfKRAS during study course. OS, overall survival; RFS, recurrence-free survival; PDAC, pancreatic ductal adenocarcinoma

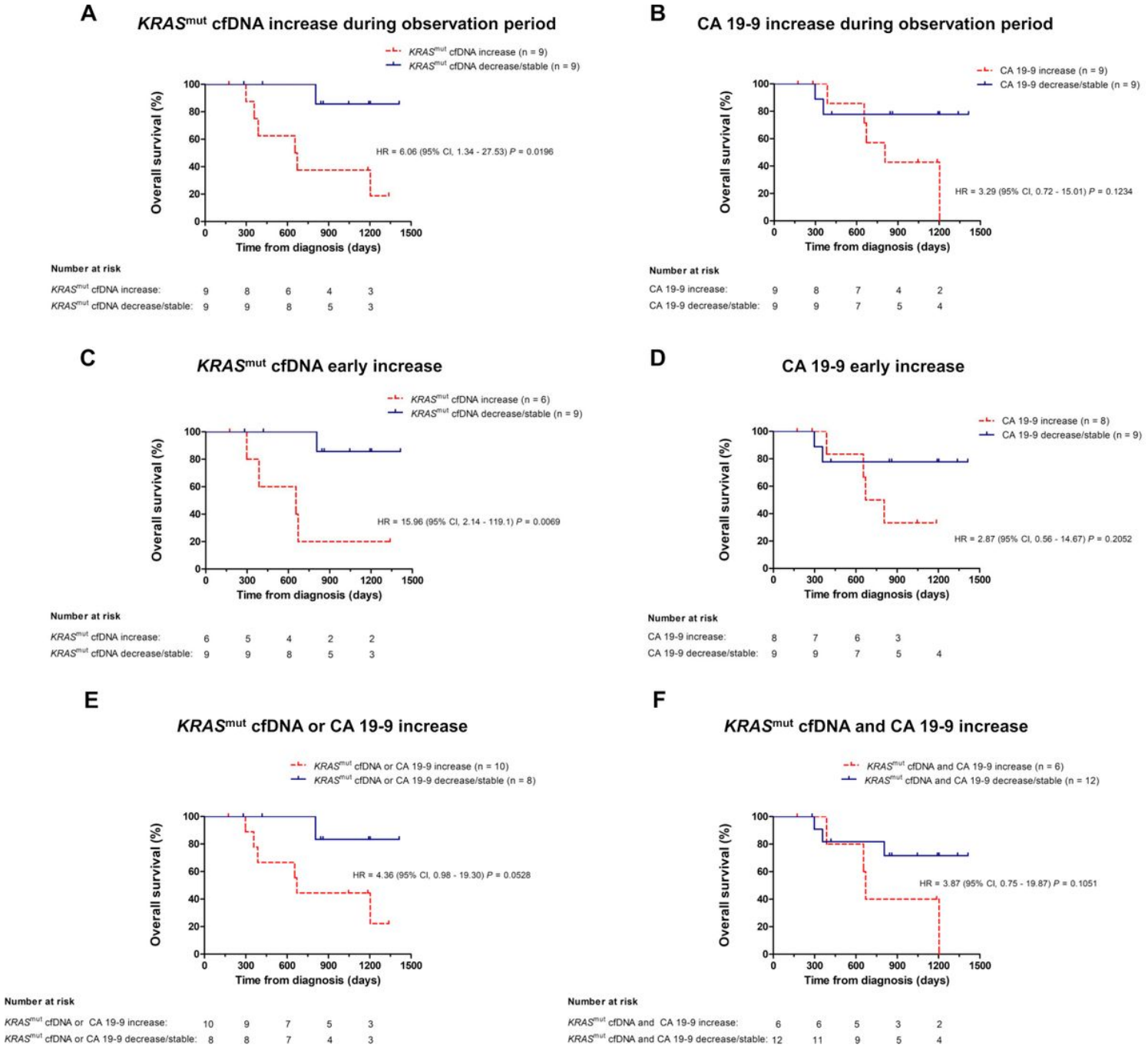

Figure 2

Association of cfKRASmut and CA19-9 dynamic changes with survival endpoints (A, B) Kaplan-Meier estimates of OS for resected PDAC patients with increase of cfKRASmut (A) or CA 19-9 (B) during observation period. (C, D) Kaplan-Meier estimates of OS for resected PDAC patients with early increase of cfKRASmut (A) or CA 19-9 (B) during observation period. Early increase was defined as increase within 6 months after surgery. (E) Kaplan-Meier estimates of OS for resected PDAC patients with combined early increase of cfKRASmut or CA 19-9. (F) Kaplan-Meier estimates of OS for resected PDAC patients with

combined early increase of cfKRASmut and CA 19-9. OS, overall survival; RFS, recurrence-free survival; PDAC, pancreatic ductal adenocarcinoma

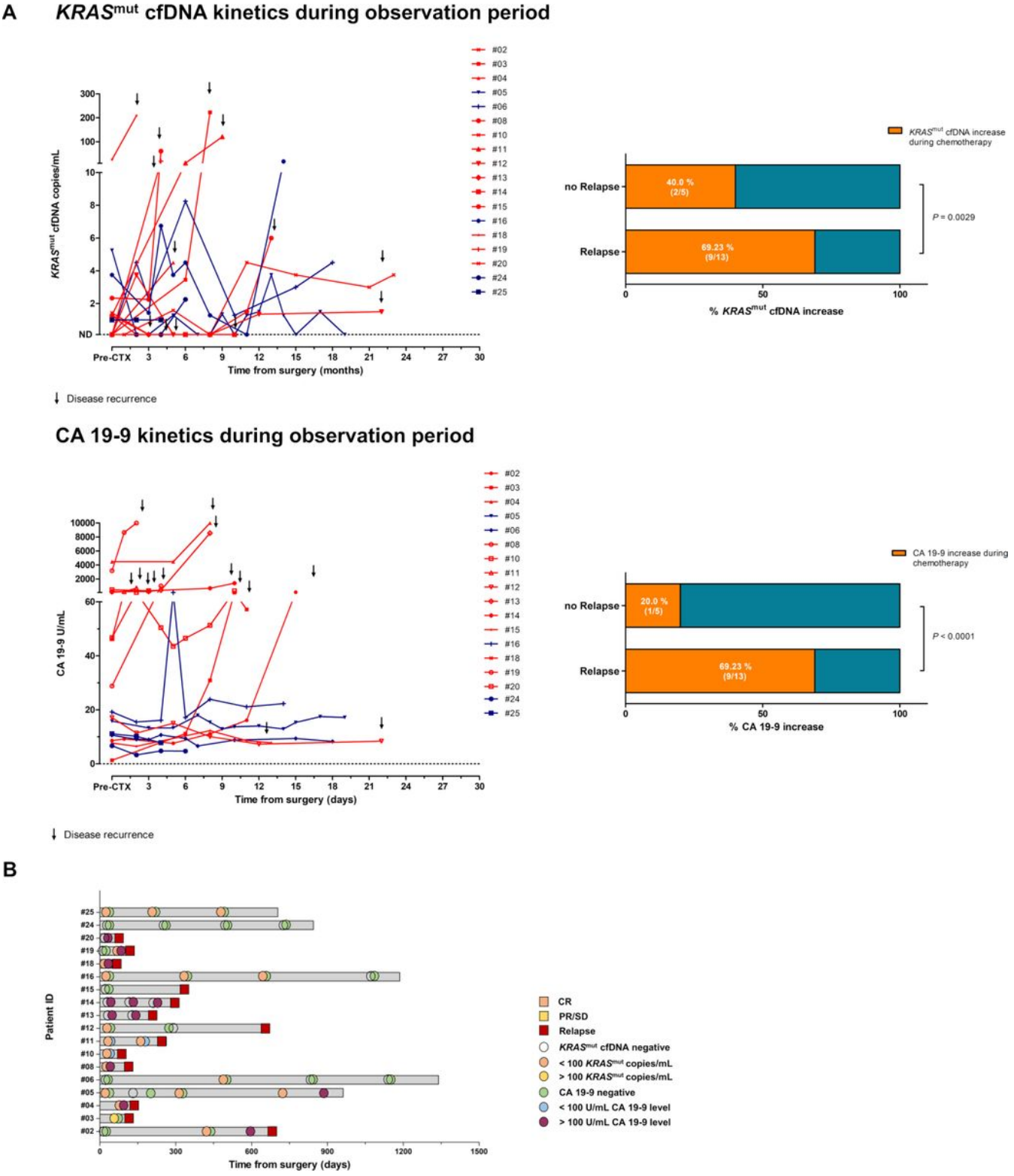

Figure 3

Longitudinal cfKRASmut and CA 19-9 monitoring (A) Top left: Absolut levels of cfKRASmut during observation period. Patients with relapse during study period are marked red. Black arrow mark the time of disease recurrence. Top right: Relapse versus non-relapse patients with increase in cfKRASmut during

observation period. Fisher’s exact test was used to test for statistical significance between the two groups. P values < 0.05 were considered significant. Bottom left: Absolut levels of CA 19-9 during observation period. Patients with relapse are marked red. Black arrows mark the time of disease recurrence. Bottom right: Relapse versus non-relapse patients with increase in cfKRASmut during observation period. Fisher’s exact test was used to test for statistical significance between the two groups. P values < 0.05 were considered significant. (B) Swimmers plot of disease course of resected PDAC patients. cfKRASmut and CA 19-9 analysis in blood were compared to clinical course of disease before and during adjuvant chemotherapy. CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.

Supplementary Files

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