

**Keywords:** pancreatic ductal adenocarcinoma; *KRAS*; cell-free DNA; biomarker; survival; treatment response

# *KRAS* mutations in cell-free DNA from preoperative and postoperative sera as a pancreatic cancer marker: a retrospective study

Yutaka Nakano<sup>1</sup>, Minoru Kitago<sup>\*1</sup>, Sachiko Matsuda<sup>1</sup>, Yuki Nakamura<sup>1</sup>, Yusuke Fujita<sup>1</sup>, Shunichi Imai<sup>1</sup>, Masahiro Shinoda<sup>1</sup>, Hiroshi Yagi<sup>1</sup>, Yuta Abe<sup>1</sup>, Taizo Hibi<sup>1</sup>, Yoko Fujii-Nishimura<sup>2</sup>, Ayano Takeuchi<sup>3</sup>, Yutaka Endo<sup>1</sup>, Osamu Itano<sup>4</sup> and Yuko Kitagawa<sup>1</sup>

<sup>1</sup>Department of Surgery, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan; <sup>2</sup>Department of Pathology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan; <sup>3</sup>Department of Preventive Medicine and Public Health, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan and <sup>4</sup>Hepato-Biliary-Pancreatic and Gastrointestinal Surgery, International University of Health and Welfare School of Medicine, 14-1-6 Kokufudai Ichikawa-shi, Chiba 272-0827, Japan

**Background:** Pancreatic ductal adenocarcinoma (PDAC) has very poor prognosis despite existing multimodal therapies. This study aimed to investigate whether *KRAS* mutations at codons 12/13 in cell-free DNA (cfDNA) from preoperative and postoperative sera from patients with PDAC can serve as a predictive biomarker for treatment response and outcomes after surgery.

**Methods:** Preoperative and postoperative serum samples obtained from 45 patients with PDAC whom underwent curative pancreatectomy at our institution between January 2013 and July 2016 were retrospectively analysed. Peptide nucleic acid-directed PCR clamping was used to identify *KRAS* mutations in cfDNA.

**Results:** Among the 45 patients enrolled, 11 (24.4%) and 20 (44.4%) had *KRAS* mutations in cfDNA from preoperative and postoperative sera, respectively. Multivariate analysis revealed that *KRAS* mutations in postoperative serum (hazard ratio (HR) = 2.919; 95% confidence interval (CI) = 1.109–5.621; *P* = 0.027) are an independent prognostic factor for disease-free survival. Furthermore, the shift from wild-type *KRAS* in preoperative to mutant *KRAS* in postoperative cfDNA (HR = 9.419; 95% CI = 2.015–44.036; *P* = 0.004) was an independent prognostic factor for overall survival.

**Conclusions:** Changes in *KRAS* mutation status between preoperative and postoperative cfDNA may be a useful predictive biomarker for survival and treatment response.

Pancreatic ductal adenocarcinoma (PDAC) is a lethal malignancy characterised by rapid progression and poor prognosis. The disease is associated with a 5-year survival rate of <10%, which is mainly attributable to aggressive tumour behaviour and late clinical detection (Siegel *et al*, 2016).

Most of the patients with PDAC carry mutations in the *KRAS* gene, which encodes a member of the RAS family of GTPases. *KRAS* mutations have been identified in 67.4% and 79.3% of formalin-fixed paraffin-embedded (FFPE) and fresh-frozen tumour samples, respectively, and in 40.8% of plasma/serum

\*Correspondence: Dr M Kitago; E-mail: dragonpegasus@keio.jp

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samples (Li *et al*, 2016). *KRAS* mutation status in various patient tissues has been identified as a prognostic biomarker for PDAC outcome (Li *et al*, 2016). Liquid biopsy is considered a helpful non-invasive test for early-stage cancer diagnosis and for the assessment of treatment responses to chemotherapy or surgery (Mori *et al*, 2005; Shinozaki *et al*, 2007; Schwarzenbach *et al*, 2011).

Nucleic acids are released into the blood circulation upon cell apoptosis or necrosis (Earl *et al*, 2015). Accordingly, increased cell turnover owing to rapid tumour progression, necrosis, and apoptosis leads to elevated levels of cell-free DNA (cfDNA) (Schwarzenbach *et al*, 2011). Liquid biopsy is a minimally invasive technology to reveal molecular biomarkers in the peripheral blood, using mostly circulating tumour (ctDNA) and circulating tumour cells (CTCs) (Mori *et al*, 2005; Shinozaki *et al*, 2007; Schwarzenbach *et al*, 2011). Liquid biopsy analysis of cfDNA, ctDNA, and CTCs from plasma or serum of cancer patients, including those with PDAC, has been widely used to detect cancer-related mutations, for early detection of disease progression, and/or for monitoring treatment response to chemotherapy (Mori *et al*, 2005; Shinozaki *et al*, 2007; Kitago *et al*, 2009; Earl *et al*, 2015; Takai *et al*, 2015; Gao *et al*, 2016). For example, Diehl *et al* (2008) reported that ctDNA is a promising source of biomarkers to follow the therapeutic course in patients with metastatic colorectal cancer, showing that patients who had detectable ctDNA after surgery generally relapsed within 1 year. The frequency of CTC detection in PDAC is generally very low (Allard *et al*, 2004), and owing to advances in DNA extraction and PCR technologies, ctDNA is more clinically useful than CTCs (Cabel *et al*, 2017). Although several retrospective studies have reported the utility of *KRAS* mutations in cfDNA from patients with PDAC (Earl *et al*, 2015; Takai *et al*, 2015), it remains unclear whether changes in the *KRAS* mutation rate between preoperative and postoperative sera can be used as an indicator for treatment responses in PDAC.

Therefore, in this study, we analysed the *KRAS* mutation status in cfDNA from preoperative and postoperative serum samples to determine whether it can serve as a biomarker to monitor the treatment response of patients with PDAC after curative resection and to predict disease outcome. The mutation status of the primary tumour was determined using FFPE tissue samples.

## MATERIALS AND METHODS

**Patients.** Patients who underwent curative pancreatectomy for PDAC at our institution between January 2013 and July 2016 were retrospectively analysed. All patients were histologically proven to have invasive ductal carcinoma. Patients who had undergone R2 operation and for whom preoperative and postoperative serum samples had not been collected were excluded. Patients with or without *KRAS* mutations identified in preoperative and postoperative sera were divided into four groups according to the pattern of *KRAS* mutations. Group 1 included patients with wild-type *KRAS* (wt*KRAS*) in both preoperative and postoperative sera (pre<sup>-</sup>/post<sup>-</sup>), Group 2 included patients with wt*KRAS* in preoperative but mutant *KRAS* (mt*KRAS*) in postoperative sera (pre<sup>-</sup>/post<sup>+</sup>), Group 3 comprised patients with mt*KRAS* in preoperative but wt*KRAS* in postoperative sera (pre<sup>+</sup>/post<sup>-</sup>), and Group 4 included patients with mt*KRAS* both in preoperative and postoperative sera (pre<sup>+</sup>/post<sup>+</sup>). All participants provided written informed consent. The study was approved by the Human Experimentation Committee of our institution and was conducted in accordance with the Helsinki Declaration of 1975. The trial was registered with the University Hospital Medical Information

Network (UMIN) Clinical Trials Registry (<http://www.umin.ac.jp/ctr/number:UMIN-000014691>).

**Clinicopathological characteristics.** Preoperative clinical variables included age, sex, presence of diabetes mellitus, family history of cancer, treatment with neo-adjuvant chemoradiotherapy (NACRT), and surgical procedure. Postoperative variables included operation time, blood loss, and complications evaluated according to Clavien–Dindo classification. In our institution, some patients diagnosed with T3 or T4 disease according to the Seventh edition of the Union for International Cancer Control (UICC) TNM classification of malignant tumours have been receiving NACRT since 2003 (Fujii-Nishimura *et al*, 2015). In addition, perioperative portal vein infusion (PVI) chemotherapy on the day of surgery has been performed since 1984 as a standard treatment to prevent liver metastasis and to improve the survival of patients subjected to potentially curative resection of pancreatic cancer (Takahashi *et al*, 1999; Aiura *et al*, 2010).

The pathologic stage of residual tumours (R) was determined according to the Seventh edition of the UICC TNM classification. R0 resections showed no tumour residues, R1 resections showed microscopically positive margins, and R2 resections still showed some gross tumours. Prognostic pathological features assessed by histology included tumour size, distal bile-duct invasion, duodenal invasion, serosal invasion, retropancreatic tissue invasion, portal vein invasion, arterial invasion, extrapancreatic nerve plexus invasion, invasion of other organs, lymph node metastasis, lymphatic infiltration, venous infiltration, and intrapancreatic nerve infiltration (Kanehara, 2009).

### Preparation of and DNA extraction from FFPE tissue samples.

Resected specimens were immediately fixed in 10% buffered formalin. The fixed specimens were serially sectioned (5-mm thickness) and embedded in paraffin within 1 week using routine methods. The paraffin sections were stained with haematoxylin and eosin. After the slides from each individual patient were reviewed, the main tumour lesion was designated by a surgical pathologist.

Ten-micrometre sections of the primary tumour were cut from each block and placed on glass slides. One section of the main tumour was stained with haematoxylin and eosin for orientation, which was confirmed by a surgical pathologist. Tiny fractions of the main tumour lesions were dissected from the 10- $\mu$ m sections macroscopically, and fractions from two or three sections were collected in sterile tubes. DNA was extracted and purified from these paraffin sections using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) (Kitago *et al*, 2004).

**Serum sample collection.** Preoperative serum samples were collected 1 day before operation or on the day of operation. Postoperative serum samples were obtained before discharge from the hospital if patients showed no signs of inflammation, such as fever and elevated levels of inflammatory indicators in laboratory tests. Twelve-millilitre blood samples were collected in Venoject II tubes (Terumo, Tokyo, Japan) and were processed immediately. To separate serum from peripheral blood cells, samples were centrifuged at 1750 g at 21 °C for 10 min, and the serum was stored at -80 °C.

**DNA extraction from serum.** cfDNA was extracted from serum using the Plasma/Serum Cell-Free Circulating DNA Purification Midi Kit (Norgen Biotek, Thorold, Ontario, Canada) according to the manufacturer's instructions. This method employs a two-column method for the isolation of high-quality, high-purity, and inhibitor-free cfDNA from fresh or frozen plasma/serum samples. Briefly, 1–4 ml of serum was used as an input volume on the first column to extract cfDNA, which was concentrated on the second

mini column into a final elution volume of 30  $\mu$ l. The cfDNA obtained was stored at  $-80^{\circ}\text{C}$ .

**Quantification of genomic DNA in FFPE and serum samples.** Gene dosage analysis was conducted by real-time quantitative PCR (qPCR) using the TaqMan RNase P Detection Reagents Kit (Applied Biosystems, Carlsbad, CA, USA). The reaction was carried out in a total volume of 20  $\mu$ l, including 2 ng of sample DNA, 1  $\mu$ l of RNase P Detection Reagents ( $20\times$ ) containing primers and probe (Applied Biosystems FAM, dye-labelled with TAMRA quencher), and 8  $\mu$ l of sterile water. The thermal cycling conditions were as follows:  $50^{\circ}\text{C}$  for 2 min,  $95^{\circ}\text{C}$  for 10 min, and 45 cycles of  $95^{\circ}\text{C}$  for 15 s and  $60^{\circ}\text{C}$  for 1 min. Each sample was assayed in duplicate in the Viia7 Real-Time PCR System (Applied Biosystems). Healthy male genomic DNA was used as a calibration standard.

**Primers and probes for peptide nucleic acid (PNA)-directed PCR clamping.** Because cfDNA mtKRAS is present in very small copy numbers and is difficult to identify among thousands of wtKRAS copies, we used the PNA method to inhibit amplification of excess non-target DNA (Taback *et al*, 2004; Kim *et al*, 2006). PNA is a synthetic nucleic acid polymer that binds the wtKRAS allele surrounding codons 12/13 and inhibits the annealing of the reverse primer, thus blocking fragment amplification. PNA-directed PCR clamping is more efficient for detecting KRAS mutations at codons 12/13 than other methods, such as direct sequencing (Taback *et al*, 2004; Kim *et al*, 2006; Araki *et al*, 2010; Han *et al*, 2016). DNA was amplified by qPCR using the following primers: KRAS, 5'-GGCCTGCTGAAAATGA-3' (forward) and 5'-AAGGCACTCTTGCTA-3' (reverse). The sequences of the FRET probe and PNA were 5'-FAM-AGCTCCAACCTACCACAAGTT-TATATTC-BHQ-1-3' and 5'-TACGCCACCAGCTCC-3', respectively. Genomic DNA (2 ng) was amplified in a 25- $\mu$ l reaction containing 1  $\mu$ M of each primer, 1  $\mu$ M of probe, 1.75  $\mu$ M of PNA, and 12.5  $\mu$ l of TaqMan gene expression master mix (Applied Biosystems). PCR was carried out in the Viia7 Real-Time PCR System using the following temperature conditions: 40 cycles at  $94^{\circ}\text{C}$  for 60 s,  $70^{\circ}\text{C}$  for 50 s, and  $58^{\circ}\text{C}$  for 50 s, and a final extension at  $72^{\circ}\text{C}$  for 60 s. Reactions were also conducted without PNA to amplify wtKRAS and to verify DNA integrity. Each sample was assayed in duplicate with positive and negative controls.

**Cell lines.** To assess the accuracy and sensitivity of PNA-directed PCR clamping, we analysed four pancreatic cancer cell lines. AsPC-1 and Capan-1 containing mtKRAS and BxPC-3 and Hs 700T containing wtKRAS were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured as recommended. PCR was conducted using 0.2 and 2 ng of genomic DNA as a template.

**Statistical analysis.** Statistical analysis was conducted using IBM SPSS statistics version 23.0 (IBM Japan, Tokyo, Japan). Clinicopathological features were compared between patients with and without KRAS mutations in postoperative sera and between the four groups with different KRAS mutation status in preoperative and postoperative sera. Categorical variables were compared by the chi-square or Fisher exact test and continuous variables were compared by the Mann-Whitney *U*-test with Bonferroni's correction for multiple comparisons. Correlation between early recurrence ( $\leq 6$  months) and cfDNA KRAS mutation status was analysed using Cohen's kappa statistics. Survival duration was calculated according to the Kaplan-Meier method, and survival curves were compared by the Wilcoxon test. A Cox proportional hazards model was used to determine independent prognostic factors among preoperative and postoperative variables. The stepwise method was used for multivariate analysis.

## RESULTS

**Validation of the PNA clamp PCR assay.** The sensitivity and accuracy of the PNA clamp method for the detection of KRAS mutation have been previously established (Taback *et al*, 2004; Kim *et al*, 2006). To validate the sensitivity of the PNA quantitative real-time PCR assay in our current study, AsPC-1 DNA was mixed with Hs 700T DNA at different ratios: 1:10, 1:100, and 1:1000, and the PNA clamp PCR assay was carried out using 2 ng of DNA from each of the mixtures. KRAS mutation could be detected at a mutant-to-wild-type DNA ratio of 1:1000. To assay the accuracy, sequence analysis was conducted for 10 primary tumours found to be either KRAS-positive ( $n=7$ ) or KRAS-negative ( $n=3$ ) by PNA clamp PCR. Except for one sample, direct sequencing of the PCR product confirmed the presence or absence of mutation as detected by PNA clamp PCR, and the KRAS mutation status of these samples as determined by PNA clamp PCR was consistent with the results of direct sequencing.

**Detection of KRAS mutations in pancreatic cancer cell lines and sera of healthy donors.** The results of PNA-directed PCR clamping applied to cancer cell lines and sera of cancer-free donors indicated that a sample could be considered positive for KRAS mutations if the  $C_t$  number was  $<35$  when 2 ng cfDNA was used.

**Patient characteristics and KRAS mutation status.** In total, 84 patients underwent curative pancreatectomy in our hospital between January 2013 and July 2016. Thirty-nine patients were excluded because of incomplete preoperative and postoperative serum samples; the remaining 45 patients were enrolled in the study. Clinicopathological characteristics of the 45 patients are presented in Table 1, and characteristics of patients with wtKRAS and mtKRAS in postoperative serum are shown in Table 2. KRAS mutations of FFPE were detected in 35 of 42 (83.3%) primary tumours. For three patients, we were unable to determine KRAS mutation status. cfDNA KRAS mutations were detected in 11 (24.4%) preoperative and 20 (44.4%) postoperative samples.

The 45 patients were divided into four groups based on the presence of KRAS mutations in preoperative and postoperative sera: 20 patients (44.4%) were assigned to Group 1 (pre<sup>-</sup>/post<sup>-</sup>), 14 (31.1%) to Group 2 (pre<sup>-</sup>/post<sup>+</sup>), 5 (11.1%) to Group 3 (pre<sup>+</sup>/post<sup>-</sup>), and 6 (13.3%) to Group 4 (pre<sup>+</sup>/post<sup>+</sup>). Clinicopathological characteristics of patients in the four groups are shown in Table 3.

**Association of KRAS mutations in preoperative and postoperative sera with survival.** There were no significant differences in disease-free survival (DFS) and overall survival (OS) between patients with and without KRAS mutations in cfDNA from preoperative serum. However, patients with mtKRAS in postoperative cfDNA showed significantly shorter DFS ( $P=0.014$ ; Figure 1A) and OS ( $P=0.044$ ; Figure 1B) than those with wtKRAS.

Survival curve analysis did not reveal significant differences in DFS (Figure 2A) and OS (Figure 2B) among the groups. However, pair-wise comparison showed that patients in Group 2 (pre<sup>-</sup>/post<sup>+</sup>) had significantly shorter DFS than those in Group 1 (pre<sup>-</sup>/post<sup>-</sup>) ( $P=0.022$ ; Figure 2C), although no difference in OS ( $P=0.071$ ; Figure 2D) was observed.

In total, 8 (17.8%) patients had early recurrence ( $\leq 6$  months), and correlation analysis revealed a significant association between early recurrence and KRAS mutations in postoperative cfDNA ( $\kappa=0.426$ ;  $P<0.001$ ). At the same time, positive and negative correlations with early recurrence were detected in Group 1 (pre<sup>-</sup>/post<sup>-</sup>) ( $\kappa=0.295$ ;  $P=0.049$ ) and Group 2 (pre<sup>-</sup>/post<sup>+</sup>) ( $\kappa=-0.340$ ;  $P=0.005$ ), respectively.

**Table 1. Clinicopathological characteristics of PDAC patients enrolled in the study (n = 45)**

Parameter	
Age, years (median (range))	70 (38–87)
Sex, n (%)	
Male	29 (64.4)
Female	16 (35.6)
Diabetes mellitus, n (%)	14 (31.1)
Family cancer history, n (%)	17 (37.8)
NACRT, n (%)	
Performed	11 (24.4)
Not performed	34 (75.6)
Surgery, n (%)	
Pancreaticoduodenectomy	25 (55.6)
Distal pancreatectomy	16 (35.6)
Total pancreatectomy	4 (8.9)
Pathological stage (UICC), n (%)	
IA	2 (4.4)
IB	0 (0.0)
IIA	8 (17.8)
IIB	35 (77.8)
III	0 (0.0)
IV	0 (0.0)
Resection status, n (%)	
R0	36 (80.0)
R1	9 (20.0)
Perioperative chemotherapy, n (%)	
PI	32 (71.1)
Adjuvant chemotherapy, n (%)	
S-1 or GEM	37 (82.2)
Postoperative hospital stay, days (median ± s.d.)	29 ± 9.47
Abbreviations: GEM = gemcitabine; NACRT = neo-adjuvant chemoradiotherapy; PDAC = pancreatic ductal adenocarcinoma; PI = portal infusion; s.d. = standard deviation; UICC = Union for International Cancer Control.	

Univariate analysis of DFS and OS identified venous, serosal, and retropancreatic tissue invasions as significant prognostic factors for poor DFS, whereas the change from wtKRAS in preoperative serum to mtKRAS in postoperative serum (Group 2, pre<sup>-</sup>/post<sup>+</sup>) was a prognostic factor for poor OS (Table 4A). Multivariate analysis revealed that serosal invasion (hazard ratio (HR) = 3.919; 95% confidence interval (CI) = 1.650–9.311; P = 0.002) and KRAS mutations in postoperative cfDNA (post<sup>+</sup>) (HR = 2.919; 95% CI = 1.109–5.612; P = 0.027) were independent prognostic factors for poor DFS, whereas neural infiltration (HR = 0.197; 95% CI = 0.044–0.876; P = 0.033) and Group 2 cfDNA genotype (pre<sup>-</sup>/post<sup>+</sup>) (HR = 9.419; 95% CI = 2.015–44.036; P = 0.004) were independent prognostic factors for poor OS (Table 4B).

**DISCUSSION**

The present study investigated whether KRAS mutations in cfDNA from serum samples taken before and after curative surgery could be a prognostic factor in patients with PDAC. To the best of our knowledge, this study was the first to focus on changes in the KRAS mutation status between cfDNA from preoperative and postoperative sera. As a result, we identified the shift from wtKRAS before curative resection to mtKRAS after resection as an independent biomarker for poor OS in PDAC patients. Moreover, serosal invasion and KRAS mutations in cfDNA from postoperative serum were significant independent prognostic

**Table 2. Clinicopathological characteristics of patients with and without KRAS mutations in postoperative serum**

Parameter	KRAS mutation status in postoperative serum		P
	Negative (n = 25)	Positive (n = 20)	
Age, years (median (range))	70 (38–80)	70 (48–87)	0.672
Sex, n (%)			0.403
Male	17 (68.0)	12 (60.0)	
Female	8 (32.0)	8 (40.0)	
Diabetes mellitus, n (%)	6 (24.0)	8 (40.0)	0.204
Family cancer history, n (%)	10 (40.0)	7 (35.0)	0.487
Procedure, n (%)			0.178
Pancreaticoduodenectomy	16 (64.0)	9 (45.0)	
Distal pancreatectomy	6 (24.0)	10 (50.0)	
Total pancreatectomy	3 (12.0)	1 (5.0)	
NACRT, n (%)			0.167
Performed	8 (32.0)	3 (15.0)	
Resection status, n (%)			0.352
R0	21 (84.0)	15 (75.0)	
R1	4 (16.0)	5 (25.0)	
Lymph node metastasis, n (%)			0.481
N0	5 (20.0)	5 (25.0)	
N1	20 (80.0)	15 (75.0)	
Stage (UICC), n (%)			0.260
IA	2 (8.0)	0 (0.0)	
IB	0 (0.0)	0 (0.0)	
IIA	3 (12.0)	5 (25.0)	
IIB	20 (80.0)	15 (75.0)	
III	0 (0.0)	0 (0.0)	
IV	0 (0.0)	0 (0.0)	
Perioperative chemotherapy, n (%)			0.315
PI	19 (76.0)	13 (72.7)	
Adjuvant chemotherapy, n (%)			0.642
S-1 or GEM	20 (80.0)	16 (72.7)	
First recurrence region			
Liver	3 (12.0)	4 (20.0)	0.229
Lung	2 (8.0)	4 (20.0)	0.131
Peritoneal	1 (4.0)	4 (20.0)	0.126
Local	3 (12.0)	2 (10.0)	0.553
Lymph node	0 (0.0)	0 (0.0)	
Abbreviations: GEM = gemcitabine; NACRT = neo-adjuvant chemoradiotherapy; PI = portal infusion; UICC = Union for International Cancer Control.			

factors for poor DFS, whereas neural infiltration and changes in the KRAS mutation status between preoperative and postoperative serum cfDNA (pre<sup>-</sup>/post<sup>+</sup>) were significant independent prognostic factors for dismal OS. The presence of KRAS mutations in postoperative cfDNA and a change from mutation-negative to mutation-positive status showed significant positive correlation with early recurrence (≤ 6 months), whereas the consistent mutation-negative state had a significant negative correlation with early recurrence. Thus these parameters are potentially useful predictive biomarkers of early recurrence and effects of therapeutic intervention after surgery. Moreover, in view of adjuvant chemotherapy after surgery, regular monitoring of the KRAS mutation status in postoperative cfDNA may be used to determine whether the regimen should be continued or changed.

The shift from mtKRAS-negative to -positive status in cfDNA after surgery is puzzling. Possible reasons may be tumour manipulation during surgery and/or cell/DNA release from residual or potentially metastatic tumours. This notion is supported by studies showing that manipulations during pancreaticoduodenectomy might contribute to the release of CTCs to the

**Table 3. Clinicopathological characteristics of patients in the four groups**

Parameter	Group 1 (pre <sup>-</sup> / post <sup>-</sup> ) (n = 20)	Group 2 (pre <sup>-</sup> / post <sup>+</sup> ) (n = 14)	Group 3 (pre <sup>+</sup> / post <sup>-</sup> ) (n = 5)	Group 4 (pre <sup>+</sup> / post <sup>+</sup> ) (n = 6)	P
Age, years (median (range))	71 (58–82)	72 (48–85)	61 (38–71)	67 (57–87)	0.927
Sex, n (%)					0.434
Male	13 (44.8)	7 (24.1)	4 (13.8)	5 (17.2)	
Female	7 (43.8)	7 (43.8)	1 (6.3)	1 (6.3)	
Diabetes mellitus, n (%)	5 (35.7)	4 (28.6)	1 (7.1)	4 (28.6)	0.239
Family cancer history, n (%)	9 (52.9)	5 (29.4)	1 (5.9)	2 (11.8)	0.755
Procedure, n (%)					0.537
Pancreaticoduodenectomy	12 (48.0)	6 (24.0)	4 (16.0)	3 (12.0)	
Distal pancreatectomy	5 (31.3)	7 (43.8)	1 (6.3)	3 (18.8)	
Total pancreatectomy	3 (75.0)	1 (25.0)	0 (0.0)	0 (0.0)	
NACRT, n (%)					0.221
Performed	5 (45.5)	2 (18.2)	3 (27.3)	1 (9.1)	
Resection status, n (%)					0.802
R0	17 (47.2)	11 (30.6)	4 (11.1)	4 (11.1)	
R1	3 (33.3)	3 (33.3)	1 (11.1)	2 (22.2)	
Lymph node metastasis, n (%)					0.033
N0	4 (40.0)	1 (10.0)	1 (10.0)	4 (40.0)	
N1	16 (45.7)	13 (37.1)	4 (11.4)	2 (5.7)	
Pathological stage (UICC), n (%)					0.008
IA	1 (50.0)	0 (0.0)	1 (50.0)	0 (0.0)	
IB	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
IIA	3 (37.5)	1 (12.5)	0 (0.0)	4 (50.0)	
IIB	16 (45.7)	13 (37.1)	4 (11.4)	2 (5.7)	
III	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
IV	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Perioperative chemotherapy, n (%)					0.870
PI	15 (46.9)	9 (28.1)	4 (12.5)	4 (12.5)	
Adjuvant chemotherapy, n (%)					0.813
S-1 or GEM	16 (44.4)	12 (33.3)	4 (11.1)	4 (11.1)	
First recurrence region					
Liver	2 (28.6)	3 (42.9)	1 (14.3)	1 (14.3)	0.821
Lung	1 (16.7)	3 (50.0)	1 (16.7)	1 (16.7)	0.523
Peritoneal	1 (20.0)	2 (40.0)	0 (0.0)	2 (40.0)	0.210
Local	3 (60.0)	1 (20.0)	0 (0.0)	1 (20.0)	0.719
Lymph node	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	

Abbreviations: GEM = gemcitabine; NACRT = neo-adjuvant chemoradiotherapy; PI = portal infusion; UICC = Union for International Cancer Control.

portal circulation (Hirota *et al*, 2010; Gall *et al*, 2014), whereas during distal pancreatectomy with the no-touch technique, cancer cells were not observed in the portal vein (Hirota *et al*, 2005). In this study, the incidence of postoperative mtKRAS was higher among patients with distal pancreatectomy than among those with pancreaticoduodenectomy; however, the difference was not significant, suggesting that the emergence of mtKRAS-positive cfDNA may not be due to tumour manipulation during pancreatectomy. According to Diehl *et al* (2008), postoperative ctDNA levels in patients with an incomplete resection did not decrease after surgery. Instead, postoperative ctDNA levels in some patients were higher than preoperative levels because the injury to the remaining tumour tissue during surgery caused DNA release. In our study, postoperative mtKRAS was more prevalent than wtKRAS in patients with R1 resection, although the difference was not significant. Another conceivable reason for the appearance of KRAS mutations after surgery may be the presence of potentially metastatic tumours undetected by preoperative imaging that released CTCs and ctDNA. However, the contribution of the above-mentioned factors to the pre<sup>-</sup>/post<sup>+</sup> mtKRAS shift remains unclear, and further genetic and biochemical studies are required to disclose mechanisms underlying changes in the cfDNA profile after curative resection.

Because the half-lives of cfDNA and ctDNA after surgery are quite short (Lo *et al*, 1999; Diehl *et al*, 2008), the reverse change, that is, from mtKRAS to wtKRAS, may be an indication of a positive outcome. Although the follow-up period in the pre<sup>+</sup>/post<sup>-</sup> group was shorter than that in the other groups, the prognosis was better. On the other hand, the presence of KRAS mutations in both preoperative and postoperative cfDNA may indicate a lack of clinical response to surgery and chemotherapy, such as NACRT or perioperative PVI.

Our study had several limitations. First, we conducted a retrospective analysis using a small number of patients from a single institution. Second, for some NACRT-treated patients, blood samples had not been collected before NACRT, and it was unclear whether NACRT had an effect on KRAS mutations in cfDNA. Prospective studies are needed to confirm our preliminary findings and to evaluate the association between changes in the mtKRAS status in cfDNA in PDAC patients and both short- and long-term responses to treatment, including chemotherapy (NACRT, PVI, and adjuvant chemotherapy) after surgery.

In conclusion, this pilot study showed that changes in the KRAS mutation status of cfDNA might have potential clinical utility as a biomarker for monitoring treatment response and predicting survival and early recurrence ( $\leq 6$  months) in PDAC. Such an

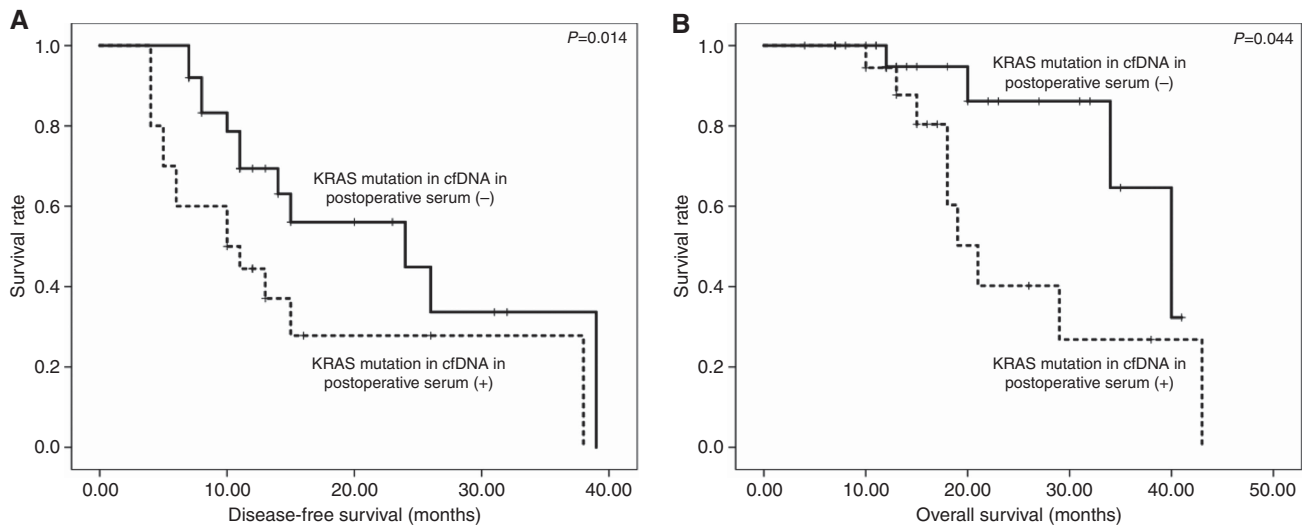


Figure 1. Correlation of KRAS mutations in cfDNA from postoperative serum with postoperative survival of PDAC patients. KRAS mutations in postoperative cell free DNA (cfDNA) significantly correlated with disease-free survival (A) and overall survival (B).

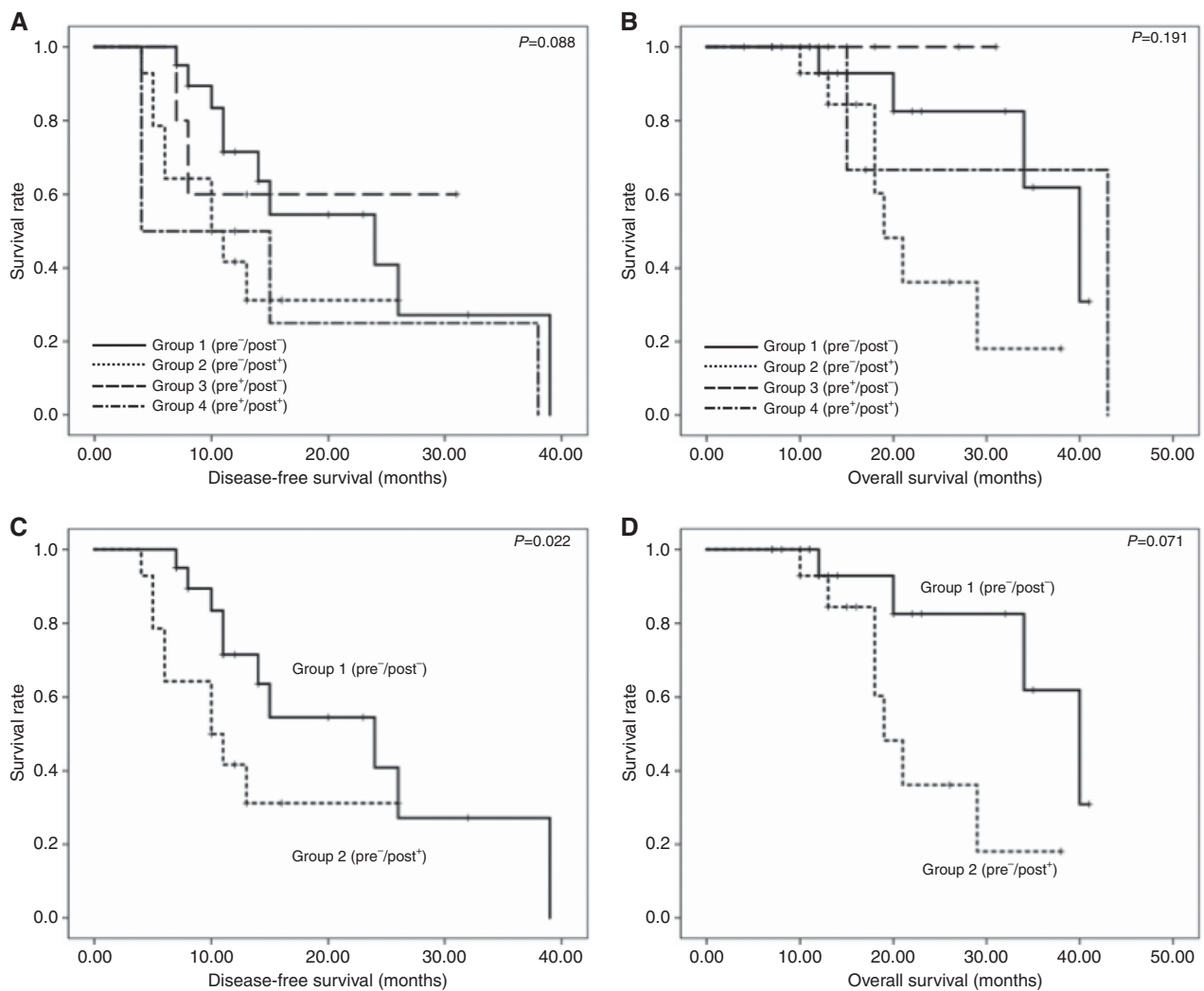


Figure 2. Correlation of the KRAS mutation status in cfDNA with postoperative survival. (A, B) Comparison of disease-free survival (DFS) (A) and overall survival (OS) (B) among the four groups of PDAC patients carrying KRAS mutations in preoperative and/or postoperative cfDNA. No significant difference was observed in DFS (A) and OS (B) between the four groups. (C, D) Comparison of DFS (C) and OS (D) between patients with wtKRAS (pre<sup>-</sup>/post<sup>-</sup>) and those shifted to the KRAS mutation status after surgery (pre<sup>-</sup>/post<sup>+</sup>).

**Table 4A. Univariate analysis of clinicopathological variables in relation to disease-free and overall survival**

Factor	Disease-free survival			Overall survival		
	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI
Age ≥70 years	0.094	0.502	0.224–1.125	0.504	0.673	0.211–2.148
Sex (male)	0.844	0.921	0.404–2.101	0.258	0.520	0.167–1.616
Diabetes mellitus (+)	0.235	1.631	0.727–3.659	0.807	1.157	0.358–3.740
Family cancer history (+)	0.701	0.844	0.356–2.001	0.079	0.291	0.073–1.154
NACRT (+)	0.338	0.591	0.202–1.732	0.970	0.975	0.262–3.626
Perioperative Adjuvant (PI) (+)	0.805	1.117	0.464–2.688	0.910	0.926	0.248–3.465
Operative time ≥450 min	0.720	1.159	0.518–2.592	0.185	2.272	0.675–7.644
Amount of bleeding ≥310 g	0.608	1.237	0.548–2.792	0.742	1.230	0.358–4.226
Complication (Clavien–Dindo ≥IIIa) (+)	0.717	0.817	0.272–2.437	0.569	0.640	0.138–2.967
Lymphatic infiltration (0 or 1)	0.127	0.503	0.208–1.215	0.432	0.613	0.181–2.074
Venous infiltration (0 or 1)	0.022	0.270	0.088–0.829	0.599	0.719	0.211–2.452
Neural infiltration (0 or 1)	0.271	0.621	0.266–1.451	0.353	0.567	0.172–1.875
Serosal invasion (+)	0.004	3.474	1.487–8.117	0.138	2.555	0.741–8.813
Retropancreatic tissue invasion (+)	0.036	4.730	1.107–20.219	0.467	1.760	0.384–8.072
Distal bile duct invasion (+)	0.443	0.709	0.295–1.708	0.313	0.509	0.137–1.890
Duodenal invasion (+)	0.981	0.990	0.451–2.175	0.102	0.364	0.109–1.223
Extrapancreatic nerve plexus invasion (+)	0.988	1.008	0.375–2.713	0.592	1.444	0.367–5.545
Vascular invasion	0.301	1.524	0.686–3.385	0.322	1.799	0.562–5.760
Lymph node metastasis	0.845	1.114	0.380–3.267	0.949	0.934	0.116–7.535
Resection margin (R0)	0.177	0.545	0.225–1.317	0.123	0.376	0.109–1.301
KRAS mutations in preoperative serum (pre <sup>+</sup> )	0.710	1.182	0.489–2.858	0.258	0.306	0.039–2.381
KRAS mutations in postoperative serum (post <sup>+</sup> )	0.052	2.200	0.993–4.873	0.060	3.184	0.951–10.656
<b>Changes in preoperative and postoperative sera</b>						
Group 1 (pre <sup>-</sup> /post <sup>-</sup> )	0.138	0.538	0.237–1.221	0.263	0.497	0.146–1.691
Group 2 (pre <sup>-</sup> /post <sup>+</sup> )	0.166	1.808	0.783–4.177	0.025	4.155	1.197–14.425
Group 3 (pre <sup>+</sup> /post <sup>-</sup> )	0.533	0.631	0.148–2.686	0.417	0.039	0.000–96.852
Group 4 (pre <sup>+</sup> /post <sup>+</sup> )	0.267	1.772	0.645–4.871	0.751	0.712	0.087–5.792

Abbreviations: CI = confidence interval; NACRT = neo-adjuvant chemoradiotherapy.

**Table 4B. Multivariate analysis of clinicopathological variables in relation to disease-free and overall survival**

Factor	Disease-free survival			Overall survival		
	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI
Neural infiltration (0 or 1)				0.033	0.197	0.044–0.876
Serosal invasion (+)	0.002	3.919	1.650–9.311			
KRAS mutations in postoperative serum (post <sup>+</sup> )	0.027	2.919	1.109–5.612			
Group 2 (pre <sup>-</sup> /post <sup>+</sup> )				0.004	9.419	2.015–44.036

Abbreviation: CI = confidence interval.

analysis is straightforward and practically relevant in clinical situations.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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