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

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**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% Cl = 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
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 responses 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 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 (Kanheara, 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-μm 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 g 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/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...
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 μl containing 2 ng of sample DNA, 1 μl of RNase P Detection Reagents (20 × ) containing primers and probe (Applied Biosystems FAM, dye-labelled with TAMRA quencher), and 8 μl of sterile water. The thermal cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 60 °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′-GGCGCTGCTGAAAATGA-3′ (forward) and 5′-AAGGCCTTCCTTGCTTA-3′ (reverse). The sequences of the FRET probe and PNA were 5′-FAM-AGGTCAACTACCAAGTTTATTTCT-BHQ-1-3′ and 5′-TACGCCCACTCGCTC-3′, respectively. Genomic DNA (2 ng) was amplified in a 25-μl reaction containing 1 μM of each primer, 1 μM of probe, 1.75 μM of PNA, and 12.5 μl 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 95 °C for 1 min. 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). Clinico-pathological 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 (≤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 Cₙ 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. Clinico-pathological 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 /-post ), 14 (31.1%) to Group 2 (pre /post ), 5 (11.1%) to Group 3 (pre / post ), and 6 (13.3%) to Group 4 (pre /post ). Clinico-pathological 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 /-post ) had significantly shorter DFS than those in Group 1 (pre /post ) (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 (≤6 months), and correlation analysis revealed a significant association between early recurrence and KRAS mutations in postoperative cfDNA (κ = 0.426; P < 0.001). At the same time, positive and negative correlations with early recurrence were detected in Group 1 (pre /post ) (κ = 0.295; P = 0.049) and Group 2 (pre /post ) (κ = −0.340; P = 0.005), respectively.
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+/post−) 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+) (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−/post+) (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 factors for poor DFS, whereas neural infiltration and changes in the KRAS mutation status between preoperative and postoperative serum were independent prognostic factors for poor 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
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 cfDNA levels in some patients with an incomplete resection did not decrease after surgery. Instead, postoperative cfDNA levels in patients with an incomplete resection did not decrease after surgery. Instead, postoperative cfDNA levels in patients with an incomplete resection did not decrease after surgery. Instead, postoperative cfDNA levels in patients with an incomplete resection did not decrease after surgery. 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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−/post−) and those shifted to the KRAS mutation status after surgery (pre−/post+).
analysis is straightforward and practically relevant in clinical situations.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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**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% Cl| P (Hazard ratio) 95% Cl |
| 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 (P) (+) | 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 >310g        | 0.608 (1.237) 0.548–2.792 | 0.742 (1.230) 0.358–4.226 |
| Complication (Clavien–Dindo ≥IIa) (+) | 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 +) | 0.710 (1.182) 0.489–2.858 | 0.258 (0.306) 0.039–2.381 |
| KRAS mutations in postoperative serum (post +) | 0.052 (2.200) 0.993–4.873 | 0.060 (3.184) 0.951–10.656 |

**Changes in preoperative and postoperative sera**

| Group 1 (pre /post +) | 0.138 (0.538) 0.237–1.221 | 0.263 (0.497) 0.146–1.691 |
| Group 2 (pre /post +) | 0.166 (1.808) 0.783–4.177 | 0.025 (4.155) 1.197–14.425 |
| Group 3 (pre +/post –) | 0.533 (0.631) 0.148–2.686 | 0.417 (0.039) 0.000–96.852 |
| Group 4 (pre +/post +) | 0.267 (1.772) 0.645–4.871 | 0.751 (0.712) 0.087–5.792 |

**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% Cl| P (Hazard ratio) 95% Cl |
| Neural infiltration (0 or 1)   | 0.033 (0.197) 0.044–0.876 | 0.004 (9.419) 2.015–44.036 |
| Serosal invasion (+)            | 0.002 (3.919) 1.650–9.311 | 0.027 (2.919) 1.109–5.612 |
| KRAS mutations in postoperative serum (post +) | 0.004 (9.419) 2.015–44.036 | 0.027 (2.919) 1.109–5.612 |

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