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Prognostic value of circulating tumour DNA in patients undergoing curative resection for pancreatic cancer

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Background: Pancreatic ductal adenocarcinoma (PDAC) is frequently diagnosed at an advanced stage, leading to a poor prognosis. Therefore, interest in the development of non-invasive biomarkers for prognostic prediction has grown rapidly. Here, we assessed the clinical implications of v-Ki-ras2 kirsten rat sarcoma viral oncogene homolog (*KRAS*)-mutated circulating tumour DNA (ctDNA) as a useful surrogate biomarker in patients with resectable PDAC.

Methods: We used droplet digital polymerase chain reaction to detect rare mutant tumour-derived *KRAS* genes in plasma cell-free DNA (cfDNA) as ctDNA. Samples were collected from 105 patients who underwent pancreatoduodenectomy for PDAC at a single institution. Overall survival (OS) was analysed according to the presence of ctDNA.

Results: Among the 105 cases, ctDNA was detected in 33 (31%) plasma samples. The median OS durations were 13.6 months for patients with ctDNA (ctDNA+) and 27.6 months for patients without ctDNA. Patients who were ctDNA+ had a significantly poorer prognosis with respect to OS ($P<0.0001$).

Conclusions: Our findings suggested that the presence of ctDNA in plasma samples could be an important and powerful predictor of poor survival in patients with PDAC. Accordingly, ctDNA detection might be a promising approach with respect to PDAC treatment.

Pancreatic ductal adenocarcinoma (PDAC) is a fatal disease that is often diagnosed at an advanced stage, leading to a poor prognosis (Poruk *et al*, 2013). This is partly attributed to the lack of suitable techniques for early detection and diagnosis. Hence, interest in the development of non-invasive biomarkers for early PDAC detection and prognostic prediction has grown rapidly.

Circulating cell-free DNA analysis, which is based on the concept of 'liquid biopsy', was recently reported as a promising prognostic biomarker in patients with various types of cancer (Gormally *et al*, 2007; De Mattos-Arruda *et al*, 2011; Mead *et al*, 2011; Kim *et al*, 2014; Kato and Janku, 2015). Cell-free DNA is derived from somatic DNA that has been released into systemic circulation following cellular necrosis and apoptosis (Kamat *et al*, 2010). Similarly, the presence of tumour-derived cell-free DNA, known as circulating tumour DNA (ctDNA), has also been studied. (Diehl *et al*, 2008; Hashad *et al*, 2012; Nygaard *et al*, 2014). However, the practical and clinical feasibility of ctDNA has not yet been determined because of the lack of suitable techniques for quantification of rare target DNA. Quantitative real-time polymerase chain reaction (qPCR) is typically used for nucleic acid quantification; however, estimation via this method requires the use of external calibrators or normalisation to endogenous controls, which consequently limits the methodological sensitivity (Hindson *et al*, 2011). The recent introduction of droplet digital PCR (ddPCR), a novel next-generation PCR technique based on nanolitre-sized water-in-oil emulsion droplet technology, allows

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highly precise nucleic acid quantification without cumbersome processing, resulting in increased sensitivity and reproducibility relative to qPCR (Hindson *et al*, 2013). Therefore, the high detection capability of ddPCR may permit the elucidation of alternative biomarkers for PDAC.

A recent study confirmed the importance of mutations in various genes, including *KRAS*, *TP53*, *SMAD4,* and *CDKN2A*, in the pathogenesis of PDAC (Biankin *et al*, 2012). Among these genes, we assumed that *KRAS* was likely to be the best-characterised tumour-related gene because of following reasons. First, among all human malignancies, PDAC exhibits the highest frequency (75%–100%) of *KRAS* mutations (Smit *et al*, 1988; Grünewald *et al*, 1989; Tada *et al*, 1996). Second, in PDAC, the most frequent *KRAS* point mutations are located in two consecutive nucleotides in codon 12 (Almoguera *et al*, 1988; Bos, 1989). Third, alterations in this gene appear to occur at an early stage of pancreatic carcinogenesis (Uemura *et al*, 2003; Rhim *et al*, 2014). Therefore, *KRAS*-mutated ctDNA represents an important potential biomarker of PDAC.

Using ddPCR, we developed a high-precision method for ctDNA detection in patients with resectable PDAC, and conducted a survival analysis based on the *KRAS* status of ctDNA.

MATERIALS AND METHODS

Patients. Matched tumour and blood-derived samples were obtained from 105 patients with PDAC. All patients underwent pancreatoduodenectomy for PDAC at the Department of Surgery, Hiroshima University Hospital, between January 2007 and May 2013. Of 105 patients, 55 (52%) were men with a median age of 69 years (range: 43–88 years). Five (5%) and 100 (95%) patients underwent standard pancreatoduodenectomy and pylorus-preserving pancreatoduodenectomy, respectively. Fifty-two (50%) patients were diagnosed with borderline-resectable PDAC according to the 2014 National Comprehensive Cancer Network (NCCN) guidelines (Tempero *et al*, 2014). Eighteen (17%) patients harboured para-aortic lymph node metastases and were histopathologically diagnosed with stage IV disease after surgery. Eighty-six (82%) patients received adjuvant gemcitabine-based chemotherapy. At the time of the last follow-up, signs of disease progression were confirmed based on imaging findings. The demographic characteristics are summarised in Supplementary Table S1.

Primary tumour samples. The samples used in this study were acquired and restricted to primary operable PDAC. After receiving ethical approval for the study, individual patients were recruited pre-operatively and provided consent through an approved process. Immediately following surgical extirpation, a special pathologist analysed the specimens macroscopically, and tumour samples were snap-frozen in liquid nitrogen. The remaining resected specimens underwent routine histopathological processing and examination. Only samples for which there was no doubt regarding the histopathological diagnosis of PDAC were included in the study. All samples were frozen at $-80\,^{\circ}C$, and genomic DNA was extracted from these cryopreserved samples using phenol-chloroform-isoamyl alcohol at a later date. Extracted DNA solutions were adjusted to approximately $100\,ng\,\mu l^{-1}$ for use in ddPCR assays.

Plasma samples. A total of 125 plasma samples were collected; these included samples from 20 healthy volunteers (11 men and 9 women, ages 27–56 years with no evidence of malignancy). Whole blood samples were collected from patients with PDAC at the time of anaesthesia induction immediately before resection. Eight millilitres of whole blood were collected in EDTA-containing tubes and centrifuged at 3000 r.p.m. (1500 **g**) at room temperature for 10 min. Plasma samples were separated from the peripheral blood cells within 4 h after collection. Subsequently, cell-free DNA was extracted from 1 ml plasma and eluted in 100 μl elution buffer with a QIAamp circulating nucleic acid kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Dawson *et al*, 2013; Sun *et al*, 2014).

TaqMan assay for specific *KRAS* amplification. For ddPCR, we used a commercially available Prime PCR for ddPCR *KRAS* kit (Bio-Rad, Hercules, CA, USA), which contained sequence-specific forward and reverse primers with dual-labelled FAM- and HEX-labelled fluorescent TaqMan probes intended to conjugate the target and reference regions, respectively (Gao *et al*, 2015).

Early reports identified several point mutations in the *KRAS* oncogene, including Gly12Asp (G12D), Gly12Val (G12V), and Gly12Arg (G12R). Other types of *KRAS* mutations (e.g., codons 13 or 61) are rarely detected in PDAC (Chen *et al*, 2010; Takai *et al*, 2015). Accordingly, these three most frequent mutations in codon 12 of *KRAS* were amplified in each sample. Other types of *KRAS* mutations were not analysed because of the limited amounts of sample plasma. Samples lacking the above-mentioned types of mutant *KRAS* were designated as wild-type in this study (Kinugasa *et al*, 2015).

Droplet digital PCR (ddPCR). The *KRAS* mutation status of each sample was analysed using a Bio-Rad QX100 ddPCR system, based on nanolitre-sized water-in-oil emulsion droplet technology. In this method, target DNA molecules are uniformly distributed across thousands of emulsified droplets, after which PCR amplification is performed in each droplet. After amplification, reactions containing one or more target DNA molecules represent the positive end-point, whereas those without target DNA molecules represent the negative end-point (Figure 1A). The number of target DNA molecules (ctDNA concentration) present can be calculated from the fraction of positive end-point reactions using Poisson statistics (Hindson *et al*, 2011).

The reaction mixture comprised 10 μl of 2 × Bio-Rad ddPCR Supermix, 1 μl of each reference and variant 20 × Bio-Rad Prime PCR for ddPCR *KRAS*, and 10 μl of template DNA in a final volume of 22 μl. The mixtures were loaded into a droplet generator cartridge with 45 μl of droplet generation oil. After placing the cartridge in the Bio-Rad QX100 droplet generator, approximately 15 000–20 000 droplets per well were generated, transferred to a 96-well reaction plate, heat-sealed with a foil seal for 5 s, and subjected to thermocycling in a Veriti 96-well thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA) under the following cycling conditions: 95 °C for 10 min; 45 cycles of denaturation at 95 °C for 30 s and extension at 55 °C for 60 s; and a final 10-min extension step at 95 °C. After cycling, the droplets were analysed immediately using a Bio-Rad QX100 droplet reader. Finally, Bio-Rad QuantaSoft analysis software was used to calculate the number of target DNA molecules from the fraction of positive end-point reactions that were plotted in a two-dimensional image (Figure 1B). Eight replicates of the same reaction were run per plasma sample to increase the reproducibility and counteract the high possibility of false-negative results because of very low levels of target DNA molecules.

Statistical analysis. Patients with PDAC were subjected to survival analyses according to the following 11 variables: age, gender, pathological differentiation, pT factor (defined by the Union for International Cancer Control (UICC)), lymph node metastasis, UICC final stage, residual tumour, pre-operative carcinoembryonic antigen value, carbohydrate antigen 19-9 (CA19-9) value, *KRAS* status of primary tumour, and presence of ctDNA. Samples were classified into two groups: ctDNA-positive (ctDNA +) and ctDNA-negative (ctDNA −). Statistical analyses were performed using the Mann–Whitney *U* test, chi-squared test, Fisher's exact test, or Spearman correlation

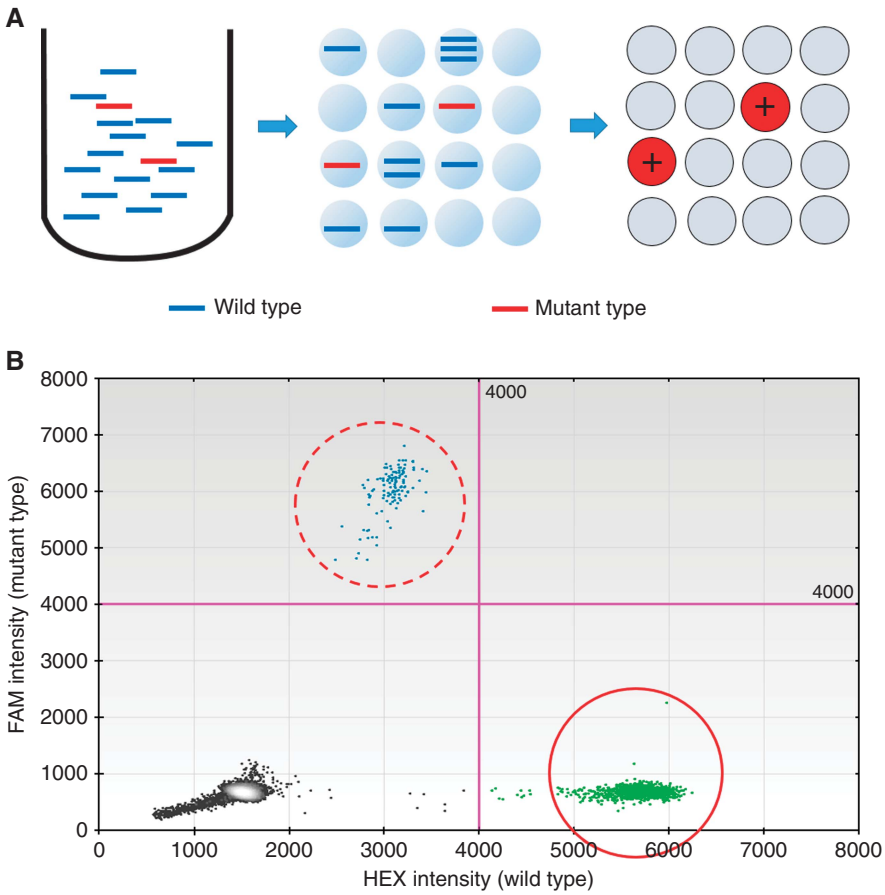

Figure 1. Overview of droplet digital PCR assay. (**A**) Schematic representation of the droplet digital PCR (ddPCR) assay, which is based on nanolitre-sized water-in-oil emulsion droplet technology. In this assay, target DNA molecules are uniformly distributed across thousands of emulsified droplets, after which PCR amplification is performed in each partitioned droplet. After amplification, reactions containing one or more target DNA molecules represent the positive end-point, whereas those without target DNA molecules represent the negative end-point. The number of target DNA molecules present can be calculated from the fraction of positive end-point reactions using Poisson statistics. (**B**) Two-dimensional histogram of ddPCR assay for *KRAS* amplification. FAM (blue) and HEX (green) fluorescence levels were plotted for each droplet. Clusters in the upper and right halves of the plot (dashed circle and solid circle) represent the positive mutant and wild-type *KRAS* end-point results, respectively.

coefficient where appropriate. Overall survival (OS) was estimated using the Kaplan–Meier method, and differences were evaluated using the log-rank test. Variables with P values less than 0.05 according to log-rank tests were retained in the multivariate Cox proportional hazards regression model. All statistical analyses were performed using JMP software, version 11.

Ethical approval to conduct human research. Approval for this study, including approval for the retrospective analysis of collected samples, was granted by the applicable Institutional Review Boards at Hiroshima University Hospital. All patients provided written informed consent.

RESULTS

Limit of detection and threshold baseline for a positive result. To evaluate the high capability of ddPCR, we performed an initial study using serial dilutions of a positive control and wild-type plasma samples from healthy controls. We found that our assay could detect a mutation prevalence of 0.01–0.1%, which corresponded to 1 mutant copy per background of 1000–10 000 wild-type copies, and we established the threshold baseline for a positive result for each types of *KRAS* mutation (Supplementary Figure S1).

Patient characteristics. A total of 105 patients with PDAC were evaluated in this study. *KRAS* mutations were identified in 86 (82%) of the 105 primary tumour specimens. The frequencies of the G12D, G12V, G12R, and wild-type *KRAS* alleles were 44 (42%), 30 (29%), 12 (11%), and 19 (18%) of 105 samples, respectively. *KRAS*-mutated ctDNA was detected in 33 (31%) of the 105 matched plasma samples at a mean ctDNA concentration of 10.1 copies per ml (range: 2.4–255 copies per ml). There was a median of 2805 copies per ml of wild-type *KRAS* fragments (range: 1212–34 320 copies per ml) in patients with PDAC, and the percentage of mutant KRAS fragments in the ctDNA + samples ranged from 0.05% to 9.5%. The frequencies of the G12D, G12V, and G12R *KRAS* subtypes were 24 (73%), 7 (21%), and 2 (6%) out of 33 ctDNA-positive samples, respectively (Figure 2A).

The *KRAS* status concordance of the tumour specimens and matched plasma samples was 100% (33/33). Two or more *KRAS* mutations did not co-exist in the same sample. Comparisons of the clinicopathological features between the ctDNA + and ctDNA − groups are summarised in Supplementary Table S2. No significant differences in the demographic characteristics and tumour burden were associated with ctDNA + results, except for adjuvant chemotherapy. No distinct increase in ctDNA concentration was observed as the disease progressed (Figure 2B).

Survival analysis. The overall follow-up period ranged from 14 to 96 months, with an average of 54 months. There was no significant

difference in OS ($P=0.18$) between patients with wild-type *KRAS* tumours ($n=19$) and those with mutant *KRAS* ($n=86$; Supplementary Figure S2a). Similarly, no differences in median OS were observed according to *KRAS* mutation subtypes (Supplementary Figure S2b). In contrast, the presence of ctDNA in plasma samples was significantly associated with a poor prognosis in both disease-free survival and OS analyses. The median disease-free survival times were 6.1 months in patients who were ctDNA+ *vs* 16.1 months in patients who were ctDNA−, and the median OS times were 13.6 months in patients who were ctDNA+ *vs* 27.6 months in patients who were ctDNA− ($P<0.001$ and $P<0.0001$, respectively). However, there were no differences in OS according to *KRAS* mutation subtype in ctDNA. As shown in Figure 3A and B, we performed Kaplan–Meier analyses of OS according to patient stratification based on their classification into the ctDNA+ or ctDNA− group followed by *KRAS* mutation subtypes of ctDNA.

In addition, univariate OS analysis of 11 independent demographic and clinicopathological variables, including the presence of ctDNA was conducted. In these univariate OS analyses, four variables (lymph node metastasis, UICC final stage, CA19-9 value, and presence of ctDNA) were identified as prognostic factors significantly associated with OS. These four variables were retained in a multivariate Cox proportional hazards regression model, through which lymph node metastasis (hazard ratio = 2.2, 95% confidence interval: 1.1–4.2, $P=0.023$), UICC final stage (hazard ratio = 2.0, 95% confidence interval: 1.1–3.5, $P=0.016$), and the presence of ctDNA (hazard ratio = 3.2, 95% confidence interval: 1.8–5.4, $P<0.001$) were identified as independent factors associated with poor prognosis. However, a high level of CA19-9 (hazard ratio = 1.2, 95% confidence interval: 0.7–2.0, $P=0.57$) did not affect long-term survival (Table 1).

In addition, subgroup analyses of OS according to resectability as defined by the 2014 NCCN guidelines were performed. Our study population included 53 (50%) patients with resectable cancers and *KRAS*-mutated ctDNA was detected in 14 (26%) patients of them. The presence of ctDNA had a significant impact on OS among patients with resectable PDAC ($P<0.001$; Supplementary Figure S3).

DISCUSSION

Pancreatic ductal adenocarcinoma is one of the most fatal diseases and has an extremely poor prognosis, which may be associated with the lack of effective screening modalities. Accordingly, a very precise predictive surrogate marker is urgently needed to clarify the pre-operative treatment strategies for this lethal disease.

In a previous study of PDAC, CA19-9 was the biomarker most commonly used as a diagnostic aid for treatment monitoring and survival prediction (Ferrone *et al*, 2006). However, elevated CA19-9 levels are also observed in many benign conditions, such

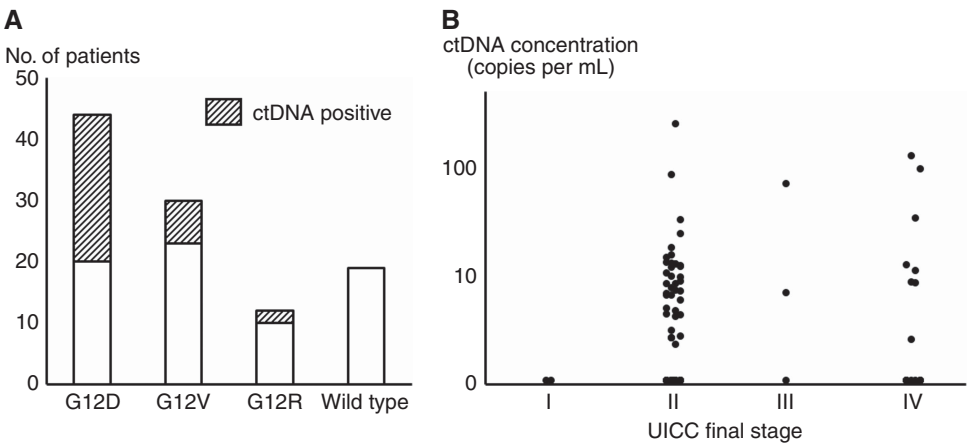

Figure 2. The results of the ctDNA detection. (**A**) Frequency of *KRAS* mutations in all primary tumour specimens and plasma samples. (**B**) Scattergram of ctDNA concentrations in all patients subdivided according to the Union for International Cancer Control (UICC) classification.

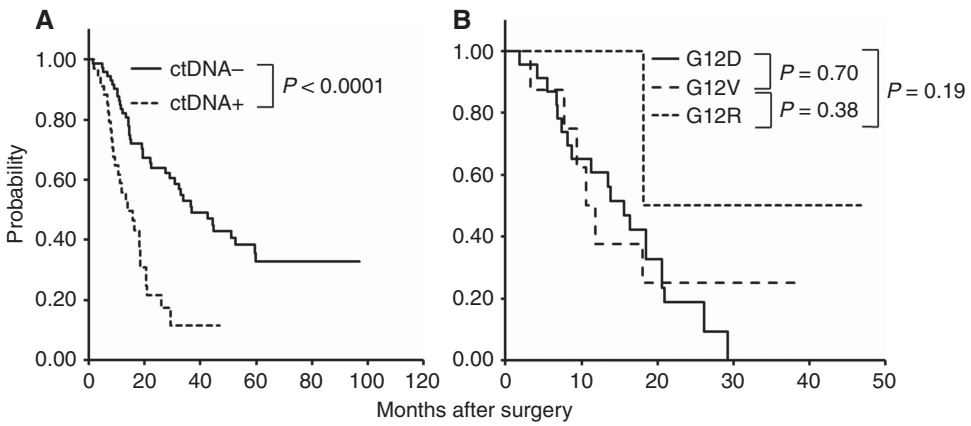

Figure 3. Overall survival curves according to the (**A**) presence of ctDNA and (**B**) *KRAS* mutation subtypes of ctDNA. Overall survival did not differ significantly according to the *KRAS* mutation subtypes of ctDNA. In contrast, significant differences in OS were observed according the categorisation of patients into ctDNA+ and ctDNA− groups.

Table 1. Univariate and multivariate analyses of OS among patients with PDAC

		Survival		Univariate analysis	Multivariate analysis	
Prognostic factors	**No. of patients**	**Median (months)**	**2-year (%)**	***P*-value**	**HR (95% CI)**	***P*-value**
Age						
<Median (69)	52	20.6	49			
⩾Median	53	18.9	51	0.62		
Gender						
Male	55	19.4	43			
Female	50	20	58	0.09		
Pathological differentiation						
Well/moderate	86	20.1	52			
Poorly	19	14.3	40	0.45		
UICC pT factor						
T1/T2	3	46.8	100			
T3/T4	102	19.4	49	0.08		
Lymph node metastasis						
No	29	38.3	40			
Yes	76	16.6	78	<0.001	2.2 (1.1–4.2)	0.023
UICC final stage						
IA/IB/IIA/IIB	84	22.6	60			
III/IV	21	14.1	14.3	<0.001	2.0 (1.1–3.5)	0.016
Residual tumour						
R0	46	24.5	57			
R1	59	12.5	37	0.07		
***KRAS* status of primary tumour**						
Wild-type	19	31	72			
Mutant	86	18.4	46	0.18		
Pre-operative CEA value						
$<5.0\,ng\,ml^{-1}$	74	21.5	47			
$\geqslant 5.0\,ng\,ml^{-1}$	31	14.5	32	0.14		
Pre-operative CA19-9 value						
$<90\,U\,ml^{-1}$	44	25	57			
$\geqslant 90\,U\,ml^{-1}$	61	19.4	41	0.023	1.2 (0.7–2.0)	0.57
Presence of ctDNA						
Negative	72	27.6	53			
Positive	33	13.6	21	<0.0001	3.2 (1.8–5.4)	<0.001

Abbreviations: CA19-9 = carbohydrate antigen 19-9; CEA = carcinoembryonic antigen; CI = confidence interval; ctDNA = circulating tumour DNA; HR = hazard ratio; *KRAS* = v-Ki-ras2 kirsten rat sarcoma viral oncogene homolog; OS = overall survival; PDAC = pancreatic ductal adenocarcinoma; UICC = Union for International Cancer Control.

as liver disease, cholangitis, and pancreatitis, and only applicable in patients with the Lewis-positive blood group (Ballehaninna and Chamberlain, 2012).

The *KRAS* status has also been reported as a useful prognostic biomarker in patients with PDAC. Some reports have described significant differences in OS between patients with wild-type *KRAS*-bearing primary tumours and those with mutant *KRAS*-bearing tumours (Tada *et al*, 1991). Relationships between codon 12 *KRAS* mutation subtypes (e.g., G12D, G12R) and shorter survival durations have also been reported (Kawesha *et al*, 2000; Ogura *et al*, 2013). These previous studies suggested the clinical feasibility of the *KRAS* mutation status of primary tumour specimens as prognostic biomarkers in patients with PDAC. However, other studies have reported conflicting results (Jimeno *et al*, 2008; Boeck *et al*, 2013; Kinugasa *et al*, 2015), and our study observed no significant differences among patients with tumours expressing wild-type and mutant *KRAS* (Supplementary Figure S2a and b). The clinical implications of these factors remain controversial and more prospective studies are needed.

In contrast, our data demonstrated that circulating *KRAS*-mutated ctDNA was associated with significantly poorer survival in both univariate and multivariate analyses, and the presence of ctDNA was the only prognostic factor pre-operatively available in this study. In addition, among patients with resectable cancer (defined by the NCCN guidelines), ctDNA + patients showed significantly poorer prognosis in OS. This finding suggested that tumour-derived ctDNA disseminated into systemic circulation in some cases in which cancer was diagnosed at an earlier stage via pre-operative imaging. Positivity for ctDNA may correlate with micrometastases that cannot be found in diagnostic imaging. Similar considerations have also been described by other researchers. Takai *et al* (2015) reported that nine patients with resectable PDAC exhibiting detectable levels of ctDNA relapsed because of formation of metastatic tumours within a short period of time, and had a very poor prognosis. Accordingly, patients who are positive for ctDNA should be considered for indications of any pre-operative options, such as neoadjuvant chemotherapy, even if radiographic evidence of disease progression is absent.

No distinct correlation between ctDNA concentrations and stage of disease was observed in this study (Figure 2B). However, it is notable that no patients with greater than 15 copies per ml of ctDNA survived beyond 2 years ($n = 12$, data not shown).

In addition, we experienced a unique case of advanced PDAC with multiple organ metastases; this patient, who had a *KRAS*-mutated ctDNA concentration exceeding 5500 copies per ml, died within 1 month after blood collection (Supplementary Figure S4). These results suggested the potential for a clearer association between a high ctDNA concentration and poorer prognosis in future larger-scale studies. In support of this potential finding, Bettegowda *et al* (2014) also reported a steady decrease in the survival rate as the ctDNA concentration increased.

The present study included only data from patients who had undergone pancreatic resection and therefore provided important, novel findings. However, this study was limited by its retrospective nature, and the collection of blood samples only at a single pre-operative time point. A comparison of pre-operative and post-operative ctDNA concentrations would be informative, as changes in ctDNA concentration after treatment may provide important information regarding the therapeutic effect, and management strategies for PDAC (Diaz and Bardelli, 2014; Sausen *et al*, 2015). Therefore, additional prospective studies will be needed, and are currently underway.

In conclusion, this exploratory study revealed the utility of *KRAS*-mutated ctDNA as a prognostic biomarker in patients with PDAC. Robust technologies, such as ddPCR, could therefore contribute to the establishment of new therapeutic strategies for PDAC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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