Diagnostic and Prognostic Values of KRAS Mutations on EUS-FNA Specimens and Circulating Tumor DNA in Patients With Pancreatic Cancer

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INTRODUCTION: **The ability of carbohydrate antigen 19-9 (CA19-9) to differentiate pancreatic cancer from other benign pancreatic lesions is unsatisfactory. This study explored the diagnostic value of *KRAS* gene mutations and plasma circulating tumor DNA (ctDNA) in patients with pancreatic cancer.**

METHODS: **The prospective cohort study comprised 149 consecutive patients with solid pancreatic lesions who underwent endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA). *KRAS* subtype mutations were analyzed by digital droplet PCR (ddPCR) in EUS-FNA histopathology tissue samples, and blood samples were sent for plasma ctDNA analysis. The final diagnosis was based on surgical resection pathology or follow-up for at least 2 years.**

RESULTS: **Adding *KRAS* mutation ddPCR increased the sensitivity and accuracy of EUS-FNA from 71.4% to 91.6% ($P < 0.001$) and 75.8% to 88.6% ($P < 0.001$), respectively. By comparison, the sensitivities of circulating biomarkers ctDNA and CA19-9 were only 35.2% and 71.2%. The area under the curve of the receiver operating characteristic curve (AUC) of EUS-FNA and *KRAS* ddPCR combination was >0.90 for distinguishing pancreatic cancer from benign lesions, whereas the AUC of EUS-FNA and CA19-9 combination was 0.83. The median survival time was significantly shorter in patients with G12D *KRAS* mutations than that in patients with other mutations (180 vs 240 days, $P < 0.001$).**

DISCUSSION: **FNA tissue sample *KRAS* mutation analysis in tissues significantly improves the diagnostic accuracy of cyto/histopathological evaluation in EUS-FNA samples. The combination of EUS-FNA and tissue sample *KRAS* ddPCR provided a more accurate method for pancreatic cancer diagnosis, superior to the combination of EUS-FNA and CA19-9/ctDNA. G12D *KRAS* mutations in pancreatic cancer were independently associated with poor overall survival.**

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A794

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BACKGROUND

Pancreatic cancer is the fourth leading cause of cancer-related death globally, with more than 60,000 estimated deaths per year in China (1,2). The prognosis for patients with pancreatic cancer has hardly improved over the past 25 years, with the median survival remaining shorter than 6 months and the 5-year overall survival remaining only 9% (3).

Currently, there are no biomarkers validated for the early detection of pancreatic cancer. Carbohydrate antigen 19-9 (CA19-9) is routinely used in the pancreatic cancer screen. Still, its ability to differentiate cancerous from benign pancreatic lesions is highly limited because CA19-9 is also elevated in patients with benign pancreatic lesions (4). Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) is the current recommended first-line technique for pancreatic cancer diagnosis, staging, and sampling (5). However, EUS-FNA is an invasive procedure, and the sensitivity and accuracy of EUS-FNA can be improved further through molecular biomarker analysis of the

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specimen obtained (6–10). One potential biomarker is the *KRAS* mutation. In pancreatic cancer, the *KRAS* mutation is the most commonly acquired mutation with reported rates of 70–95% (11). The *KRAS* gene encodes the p21 RAS protein, a small guanosine triphosphatase (GTPase). This point mutation of *KRAS* is involved in pancreatic carcinogenesis, uncontrolled proliferation, and invasion of pancreatic cancer cells (12).

When molecular mutation detection in primary pancreatic cancer is limited by the difficulty of obtaining tumor tissues, biomarkers in circulation provide a noninvasive approach that can be more widely available (13). Recently, mutant circulating tumor DNA (ctDNA) has been explored as a biomarker. The concept underlying this approach, also called "liquid biopsies," is that dying cancer cells release DNA into body fluids, such as plasma, so the DNA associated with cancer can be identified in those bodily fluids (14,15). ctDNA is a broadly applicable, sensitive, and specific biomarker used for various clinical and research purposes in patients with multiple different types of cancer (16). The fraction of patients with detectable plasmatic ctDNA and its concentration increase with adenocarcinoma stage (17). Therefore, detecting *KRAS* mutations in ctDNA can potentially be used as a diagnostic tool offering blood-based pancreatic cancer detection.

To date, it is not clear whether measurement of *KRAS* mutations in ctDNA or detection of *KRAS* mutations in EUS-FNA samples offers higher sensitivity and accuracy in the early diagnosis of pancreatic cancer. We analyzed the *KRAS* gene mutations in EUS-FNA samples from primary pancreatic cancer and the matched circulating biomarkers (ctDNA *KRAS* and CA19-9) in the same patient to address these questions. Then, we assessed the diagnostic values of *KRAS* gene mutations in EUS-FNA samples and plasma ctDNA and the prognostic value of *KRAS* gene mutations in pancreatic cancer (Figure 1). This study's primary aim was to explore the utility of detecting the KRAS gene mutation to supplement EUS-FNA evaluation of pancreatic masses. The secondary study aim was to compare the diagnostic values of *KRAS* mutations in EUS-FNA tissue samples with the matched circulating biomarkers (ctDNA KRAS and CA19-9) in the same patients. The tertiary study aim was to explore the predictive value of *KRAS* gene mutations in advanced pancreatic adenocarcinoma prognosis.

METHODS

Patients

The Tongji Medical College of Huazhong University of Science and Technology Review Board approved this prospective cohort study (IRB ID: TJ-C20140717) of *KRAS* mutation analysis in 149 consecutive patients who underwent EUS-FNA of pancreatic masses between September 2014 and May 2019 at the Endoscopy Center of Tongji Hospital. All patients provided written informed consent for procedures and tests associated with the study. The inclusion criteria were age >18 years, the presence of a solid mass lesion was confirmed by at least 1 imaging modality and was located within the pancreas, and mass size >1 cm. Exclusion criteria included (i) severe anemia (hemoglobin <60 g/L for chronic anemia and hemoglobin <70 g/L for acute anemia); (ii) pregnancy; (iii) coagulopathy (international normalized ratio >1.5), thrombocytopenia (platelet count <50,000/mm^3), and acute pancreatitis within the previous 2 weeks; or (iv) intervening structures prohibiting needle access.

EUS-FNA technique

EUS-FNA was performed under deep sedation according to the principles of monitored anesthesia care. The patients were anesthetized with the intravenous administration of propofol (2 mL/kg). All patients received oxygen during the procedure, and their blood pressure and heart rate were monitored (18). All EUS procedures were performed by an experienced endosonographer (B.C.) who had completed more than 1,000 procedures. An Olympus linear echoendoscope (GF-UCT 260 or GF-UCT 240; Olympus, Tokyo, Japan) and Pentax linear echoendoscope (EG-3870UTK; Pentax, Tokyo, Japan) were used. A 22-gauge EchoTip Ultra needle (Cook Endoscopy; Cook Medical, Bloomington, IN) was advanced into lesions with real-time EUS visualization. The endosonographer maneuvered the needle back and forth 20 times within the lesion (4 passes for each patient), applying minimal negative pressure by pulling the needle stylet slowly and continuously. If no specimen was obtained, continuous suction was applied with a 5–10-mL syringe to obtain a specimen. The operator immediately evaluated the samples aspirated from all 149 patients to determine whether the specimen was sufficient according to the results of the macroscopic onsite evaluation (19). Aspirated material from each patient was separated into 2 parts: one pass for cyto/histopathological evaluation and another pass for *KRAS* point mutation analysis. The material for cyto/histopathological analysis was immediately fixed in 10% formalin in a standard specimen bottle, centrifuged, and then embedded in paraffin. Sections were then stained by hematoxylin and eosin and by immunohistochemical staining if necessary (Figure 2). The material for *KRAS* analysis was sent to laboratory for DNA extraction immediately. Total DNA was extracted from the fresh specimens using the DNeasy Blood & Tissue Kit (Qiagen), according to the manufacturer's instructions.

Extraction of cell-free ctDNA in plasma

Each collected blood sample (5 mL) was centrifuged at 3,500 rpm for 15 minutes at 4°C within 3 hours. Then, it was stored in plasma at −80°C for further use. DNA was extracted from the plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen), according to the manufacturer's instructions. The DNA quantity was assessed by using the Qubit dsDNA HS (high sensitivity) Assay Kit (Thermo Fisher), according to the manufacturer's instructions.

Digital PCR

The sample was partitioned into 20,000 droplets by using droplet digital PCR (ddPCR) (QX200; Bio-Rad, Hercules, CA) (20,21). The DNA was concentrated and distributed among these droplets randomly. The authors tested for 3 types of *KRAS* mutations (G12V, G12R, and G12D) with PrimePCR products (Bio-Rad) for ddPCR (cat 1863115, 1863112, and 1863113) because these *KRAS* mutations encompass nearly 90% *KRAS* mutations in pancreatic cancer (12) (as shown in Figure 3a–d). Reactions were performed in 20 mL of reaction serum, which consisted of extracted DNA (5 mL), target primer mix (FAM) (1 mL), reference primer/probe mix (HEX) (1 mL), *KRAS* mutation droplet PCR supermix (10 mL), and distilled water (3 mL). PCR reactions were run on C1000 Touch thermal cycler incubating plates (Bio-Rad) at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds, followed by 10-minute incubation at 98°C. Negative controls without serum ctDNA showed no positive signal. All samples were analyzed in duplicate,

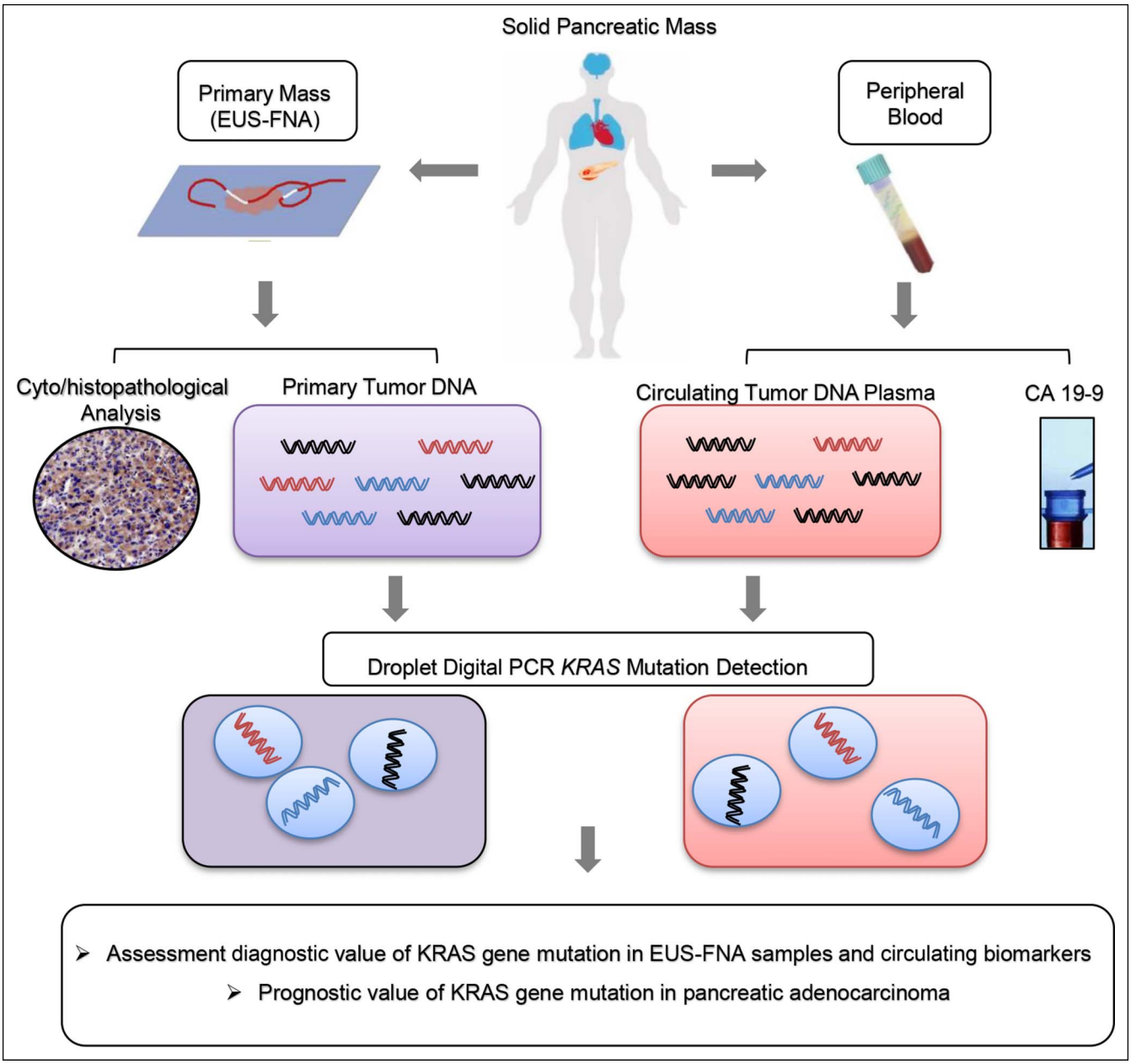

Figure 1. Schematic diagram of digital PCR analysis for KRAS mutations in EUS-FNA specimens and ctDNA samples. ddPCR analyses were performed for tumor specimens obtained from patients with pancreatic cancer using either EUS-guided FNA cytology specimens or ctDNA from blood plasma. *KRAS* mutations were evaluated for potential clinical utility or as prognostic indicators. ctDNA, circulating tumor DNA; ddPCR, digital droplet PCR; EUS-FNA, endoscopic ultrasound-guided fine-needle aspiration.

and variations were set at <5%. The detection rate was set at >0.001%. Schematic and flowdiagram of digital PCR in EUS-guided FNA cytology and ctDNA specimen analyses is shown in Figure 1.

Final diagnosis

The final diagnosis was based on a pathological examination of a surgical resection specimen (29 patients) or clinical/imaging follow-up for at least 2 years when surgical resection was not indicated because of a benign diagnosis or malignant advanced or metastasized disease. 86.7% (91/105) patients with pancreatic cancer were unresectable (III–IV stage). If signs of malignancy were absent at the end of follow-up (disease regression or no evidence of disease progression), those patients were diagnosed as nonmalignant pancreatic masses. The case was considered malignant if clinical/imaging follow-up indicated the progression or metastasis of lesions with malignant symptoms, such as weight loss, anemia, or death. Pancreatic ductal adenocarcinoma and pancreatic acinar cell carcinoma were defined as malignant diseases. Chronic pancreatitis, autoimmune pancreatitis, and pancreatic tuberculosis were defined as nonmalignant diseases. Samples that were considered malignant or suspicious for malignancy were categorized as positive for malignancy, whereas samples that were considered

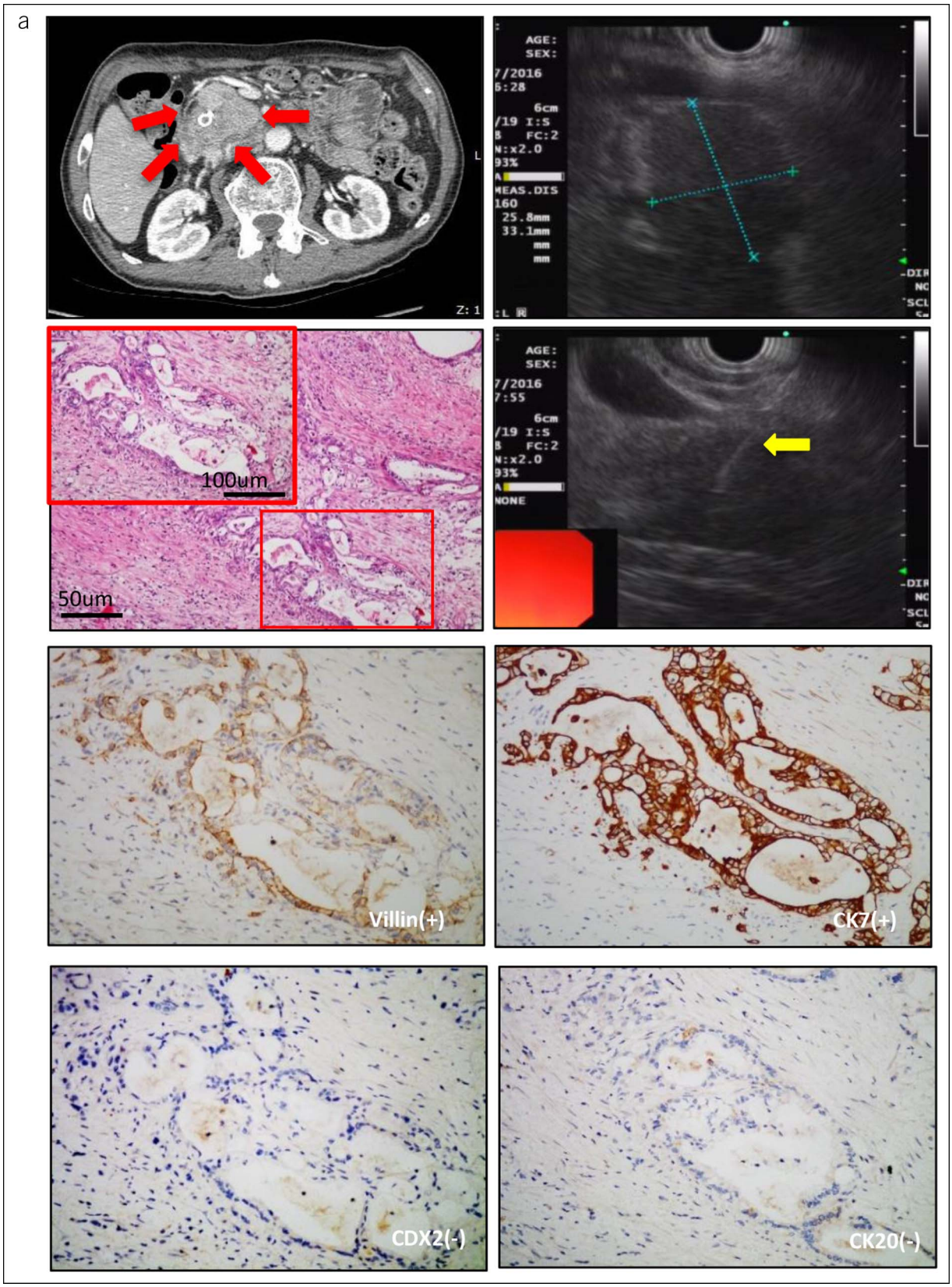

Figure 2. Histopathological features in a representative patient with pancreatic adenocarcinoma. (**a**) A representative patient with pancreatic cancer (2.58 × 3.31 cm) in computed tomography (red arrow) and EUS-FNA (yellow arrow), which expressed villin(+), Ki67(+), CDX2(−), and CD20(−). EUS-FNA, endoscopic ultrasound-guided fine-needle aspiration.

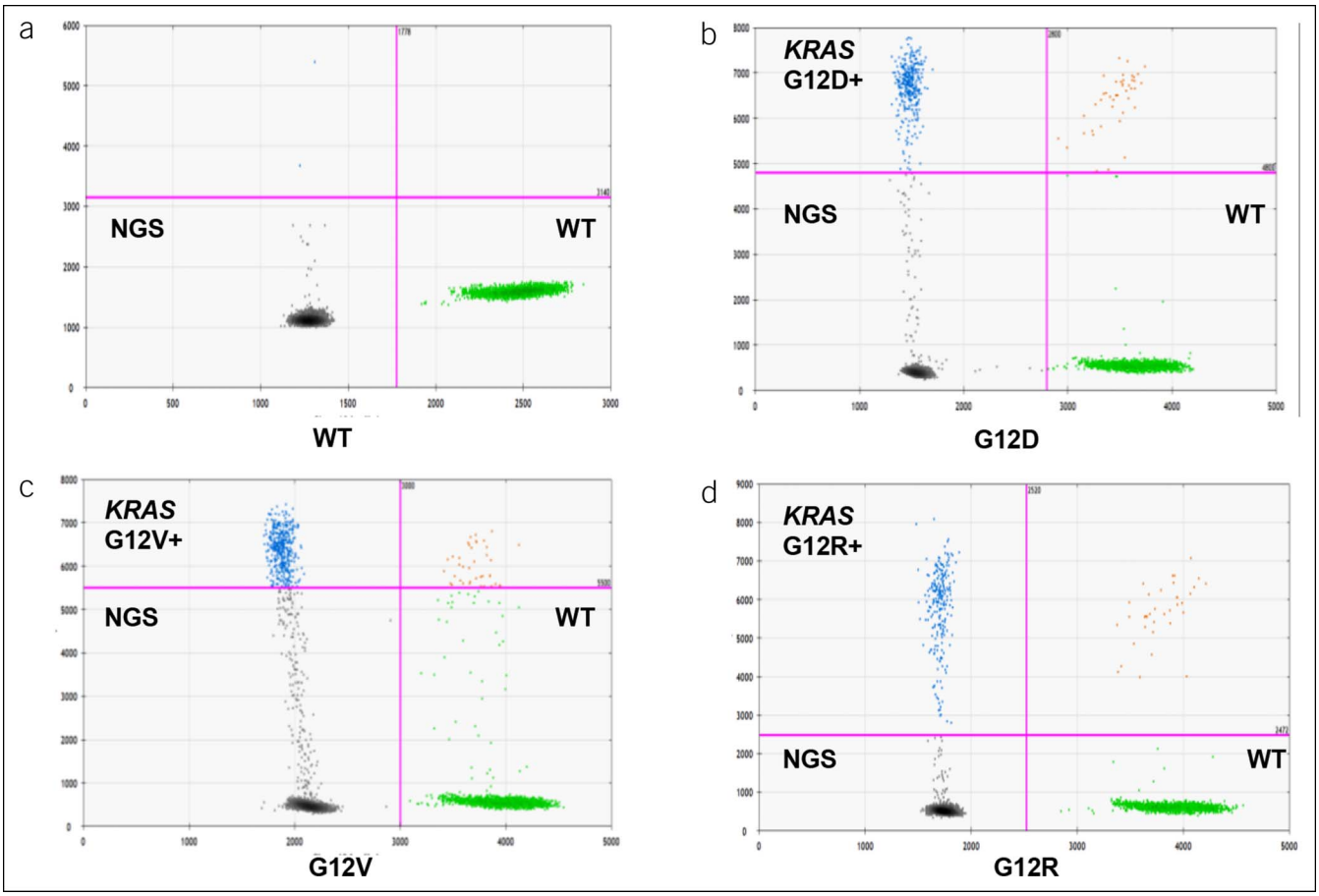

Figure 3. Digital PCR *KRAS* mutation analysis of ctDNA in representative patients with pancreatic adenocarcinoma. (**a**–**d**) Distribution of droplets is visualized using a heat map. Droplet threshold are shown as pink lines. Signal detected in the channel 1 represents DNA positive for a *KRAS* 12 (G12D, G12V, and G12R) mutation. Signal detected in channel 2 represents DNA positive for *KRAS* WT amplification in which ctDNA mutants were nondetectable. ctDNA, circulating tumor DNA; NGS, next-generation sequencing; WT, wild type.

benign or atypical were categorized as negative for malignancy (19,22).

Statistical analysis

Continuous variables are expressed as medians and ranges. Incidences and concordance between groups were compared by using the Fisher exact test or McNemar test where appropriate. All analyses were performed with SAS version 9.2. A *P* value of 0.05 was considered statistically significant.

RESULTS

Patients' characteristics

The authors prospectively evaluated 157 patients included in this study, of which 5 patients withdrew and 3 patients were lost to follow-up. Therefore, the authors analyzed 149 patients, including 105 patients with pancreatic cancer (age 58.38 ± 11.01 years; men 69/105 [65.71%]) (14 cases of whom had TNM stage I–II cancers and 91 of whom had stage III–IV cancers) and 44 cases with nonmalignant pancreatic masses (age 52.66 ± 13.81 years; men 36/44 [81.82%]) (Table 1). These 105 pancreatic cancer cases consisted of 102 pancreatic ductal adenocarcinoma cases and 3 diagnosed acinar cell carcinoma cases (Table 1). These nonmalignant 44 cases consisted of 26 autoimmune pancreatitis, 10 chronic pancreatitis, and 3 pancreatic tuberculosis. The final diagnoses were based on evaluating surgical pathology (n = 29) and clinical courses after 2-year follow-up (n = 105). One case of chronic pancreatitis with a *KRAS* G12D mutation was found to be malignant during follow-up.

Diagnostic value of *KRAS* gene mutations with EUS-FNA specimens in pancreatic adenocarcinoma

Next, we analyzed the *KRAS* gene mutations in codon 12 in EUS-FNA samples from primary pancreatic adenocarcinoma (n = 105, Figure 2, a representative patient) using the digital droplet polymerase chain reaction (ddPCR, Figure 3a–d). The sensitivity, specificity, PPV, NPV, accuracy, and ROC AUC of the EUS-FNA alone were 71.4%, 86.4%, 92.6%, 55.9%, 75.8%, and 0.765, respectively (Table 2 and Figure 4a), whereas these values of the EUS-FNA with ddPCR *KRAS* mutation analysis were 91.6%, 80.9%, 92.5%, 79.1%, 88.6%, and 0.927, respectively (Table 2 and Figure 4b). The sensitivity and accuracy of EUS-FNA diagnosis were increased from 71.4% to 91.6% ($P < 0.001$) and 75.8% to 88.6% ($P < 0.001$), respectively, when *KRAS* mutation ddPCR analysis was added to standard EUS-FNA assessment. Our study demonstrated that *KRAS* mutation analysis significantly improves the sensitivity and accuracy of EUS-FNA

Table 1. Patients' characteristics (N = 149)

Characteristics	Pancreatic cancer[a] (n = 105)	Nonmalignant pancreatic mass[b] (n = 44)
Age, mean ± SD	58.38 ± 11.01	52.66 ± 13.81
Sex, n (%)		
Male	69 (65.71)	36 (81.82)
Female	36 (34.29)	8 (18.18)
Location of mass, n (%)		
Head/ uncinate	70 (66.67)	38 (86.36)
Body/tail	35 (33.33)	6 (13.64)
CA19-9, n (%)		
≥37 U/mL	75 (71.43)	10 (22.73)
<37 U/mL	30 (28.57)	34 (77.27)
CEA, n (%)		
≥10 ng/mL	32 (30.48)	10 (22.73)
<10 ng/mL	73 (69.52)	34 (77.27)
Surgery, n (%)		
Yes	24 (22.86)	5 (11.36)
No	81 (77.14)	39 (88.64)

CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen.
[a]Pancreatic cancer including 102 ductal adenocarcinoma and 3 diagnosed acinar cell carcinoma.
[b]Nonmalignant pancreatic mass including 26 autoimmune pancreatitis, 10 chronic pancreatitis, and 3 pancreatic tuberculosis.

in pancreatic adenocarcinoma. Detecting *KRAS* gene mutations in FNA samples thus improved the diagnostic accuracy of pancreatic adenocarcinoma with EUS-FNA compared with currently available methods.

Diagnostic value of *KRAS* gene mutations in ctDNA and serum CA19-9

Aiming for a noninvasive method for detecting *KRAS* mutations, the authors also conducted a *KRAS* mutation analysis of ctDNA in all matched plasma samples. The concordance of results obtained from EUS-FNA samples and plasma samples was evaluated. As shown in Table 2 and Figure 4, the sensitivity, accuracy, and ROC AUC of *KRAS* mutations in EUS-FNA samples were 91.6%, 88.6%, and 0.889, whereas the respective values of *KRAS* mutation in ctDNA were 35.2%, 51.0%, and 0.683. In the pancreatic adenocarcinoma group, *KRAS* gene mutations were found in 88 (83.8%) of 105 cases in primary cancer, whereas only 37 cases (35.2%) of *KRAS* mutation were found in ctDNA ($P < 0.001$, χ^2 test, Table 2). The accuracy of noninvasive ctDNA *KRAS* in detecting pancreatic adenocarcinoma was thus not as high as EUS-FNS *KRAS* mutation analysis ($P < 0.0001$, Table 2). Although ctDNA *KRAS* mutation detection showed a tendency of higher positivity in patients with III/IV pancreatic cancer than in patients withI/II pancreatic cancer, there was no significant difference in sensitivity between different stages (28.6% vs 42.0%, $P = 0.506$) (see Supplementary Figure S1, Supplementary Digital Content 1, http://links.lww.com/CTG/A794). The sensitivity, accuracy, and ROC AUC of CA19-9 alone were 71.2%, 73.0%, and 0.683. In addition, the ROC AUC of EUS-FNA and CA19-9 combination was 0.830, which was still inferior to the combination of EUS-FNA and tissue sample ddPCR (Figure 4).

Prognostic value of *KRAS* gene mutations in pancreatic adenocarcinoma

The authors analyzed the effect of G12D, G12V, and G12R mutations in the following Kaplan–Meier study. The median survival time (MST) was significantly shorter in patients with G12D mutations (180 days) compared with patients with other mutations (240 days) in their EUS-FNA tissue samples and ctDNA samples (log-rank test, $P = 0.001$ and $P = 0.0008$, respectively) (Figure 5a,b). By contrast, the MST was not found to be significantly different between the patients with wild-type *KRAS* (240 days) and those with *KRAS* mutations (210 days) in their EUS-FNA tissue samples and ctDNA sample (log-rank test, $P = 0.7088$ and $P = 0.3076$, respectively) (Figure 5c,d).

Furthermore, univariate analysis demonstrated that the G12D *KRAS* mutation in both EUS-FNA tissue samples (hazard ratio [HR], 1.94; 95% confidence interval [CI], 1.12–3.36, $P < 0.0001$) and ctDNA (HR, 1.579; 95% CI, 1.383–3.520, $P < 0.0005$) was a significant factor for poor survival. Multivariate analysis demonstrated that the G12D mutation in both EUS-FNA tissue samples (HR, 1.495, 95% CI, 1.325–1.753, $P = 0.0010$) and ctDNA (HR, 1.417, 95% CI, 1.199–2.870, $P = 0.0199$) was independently associated with poor overall survival (Table 3). The

Table 2. Overall accuracy of circulating biomarkers in comparison of EUS-FNA for the diagnosis of pancreatic cancer (n = 105)

	Primary tumor		Circulating biomarkers		
	EUS-FNA	EUS-FNA + ddPCR (*KRAS*)	ctDNA (*KRAS*)	CA19-9	EUS-FNA + CA19-9
Sensitivity (%)	71.4	91.6	35.2	71.2	79.6
Specificity (%)	86.4	80.9	88.6	78.3	75.9
PPV (%)	92.6	92.5	88.1	91.2	85.4
NPV (%)	55.9	79.1	36.4	46.7	71.0
Accuracy (%)	75.8	88.6	51.0	73.0	79.5
ROC AUC	0.765	0.889	0.614	0.683	0.830

CA19-9, carbohydrate antigen 19-9; ctDNA, circulating tumor DNA; EUS-FNA, endoscopic ultrasound-guided fine-needle aspiration; NPV, negative predictive value; PPV, positive predictive value; ROC AUC, area under the curve of the receiver operating characteristic curve; combination: the combination of EUS-FNA and CA19-9.

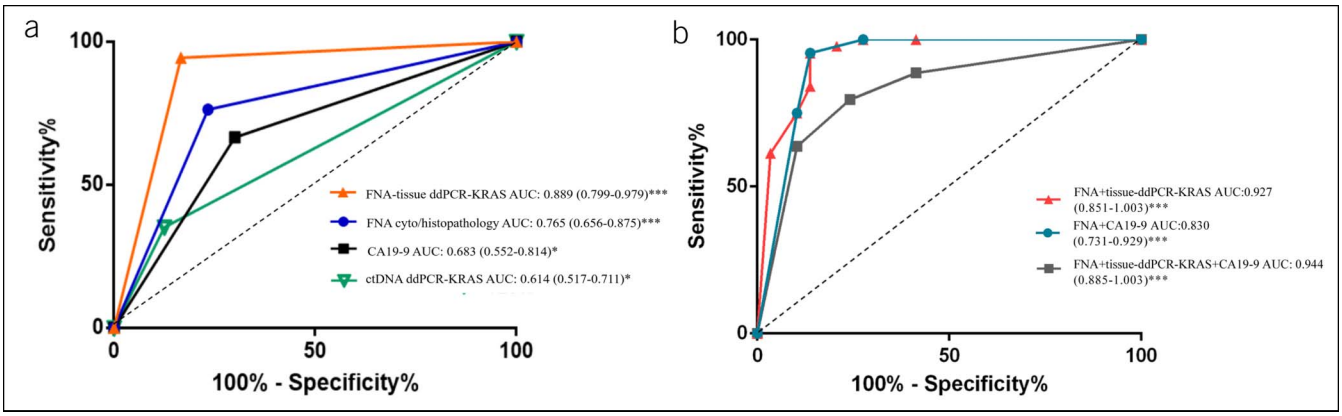

Figure 4. Diagnostic performance of EUS-FNA, CA19-9, ctDNA, and FNA specimen *KRAS* mutation detection either alone or in combination for distinguishing patients with pancreatic cancer (n = 105) from control patients (n = 44). *KRAS* mutation was determined using ddPCR. ROC curves: *X*-axis, 1-specificity; *Y*-axis, sensitivity. CA19-9, carbohydrate antigen 19-9; ctDNA, circulating tumor DNA; ddPCR, digital droplet PCR; EUS-FNA, endoscopic ultrasound-guided fine-needle aspiration; AUC, area under the curve.

following factors were analyzed as possible risk factors for survival: age, sex, TNM stage, location of masses, tumor size, carcinoembryonic antigen, CA19-9, and *KRAS* mutations (G12D, G12V, and G12R). In univariate analysis, both baseline CA19-9 and ctDNA *KRAS* were associated with overall survival, which was expected given the known positive correlation between the 2 variables.

DISCUSSION

Our study explored 3 crucial concerns on the diagnosis of pancreatic cancer. First, we evaluated whether the use of *KRAS* mutation analysis is able to improve the efficacy of pancreatic cancer diagnosis in EUS-FNA samples. Although intraepithelial neoplasia-originated cancer may take up to 10 years to develop, it only takes approximately 1 year for the pancreatic adenocarcinoma to progress from stage I to stage IV (23). Currently, cyto/histopathological evaluation of EUS-FNA samples is one of the most useful methods for the early diagnosis of pancreatic masses. However, previous studies have shown that the sensitivity, specificity, and accuracy of EUS-FNA in the diagnosis of pancreatic cancer range from 64 to 94%, 71% to 100%, and 78% to 95%, respectively (24–27), indicative of low stability. Our findings demonstrated that ddPCR analysis of *KRAS* mutations significantly improved the cyto/histopathological sensitivity and accuracy in EUS-FNA samples over currently available methods.

Second, we compared noninvasive ctDNA and EUS-FNA in detecting pancreatic cancer and found that the accuracy of *KRAS* mutation analysis through noninvasive ctDNA *KRAS* is inferior to that of *KRAS* mutation analysis in EUS-FNA samples. These findings indicated that *KRAS* mutations in plasma ctDNA, also termed as "liquid biopsy," might not be sufficient to complement the use of EUS-FNA in the diagnosis of pancreatic cancer. In addition, *KRAS* mutation ddPCR analysis in tissues significantly improves cyto/histopathological sensitivity and accuracy in EUS-FNA samples. In conclusion, combining *KRAS* mutation ddPCR and cyto/histopathological analysis in EUS-FNA samples might be more clinically valuable than blood tests for diagnosing pancreatic cancer.

To date, cyto/histopathological evaluation of EUS-FNA samples is one of the most useful methods for the early diagnosis of pancreatic masses. Conversely, ctDNA is derived from apoptosis and necrosis of tumor cells, characteristic of advanced-stage disease (28). Very few studies have reported the concordance of results between tumor and plasma samples for pancreatic cancer. In this sense, it is challenging to assess the actual diagnostic sensitivity and specificity of previous analyses. In this study, we demonstrated that the sensitivity, specificity, and accuracy of KRAS mutations in EUS-FNA samples were 71.4%, 86.4%, and 75.8%. Because these values of the *KRAS* mutation in ctDNA were 35.2%, 88.6%, and 51.0%, the accuracy of *KRAS* mutation analysis in EUS-FNA samples was higher than that in a noninvasive blood-based ctDNA *KRAS* mutation in detecting pancreatic cancer. Identification of early driver mutations in blood samples with "liquid biopsy" (16) does not alter cyto/histopathological evaluation of EUS-FNA samples. However, detection of circulating biomarkers combination (ctDNA and CA19-9) is also promising, which may complement other diagnostic techniques to diagnose pancreatic cancer.

We identified *KRAS* mutations in 35.2% of ctDNA in plasma samples from patients with pancreatic cancer, which is consistent with the data of *KRAS*-positive ctDNA in previous studies, specifically 39% (29), 35.3% (30), 32% (25), and 26% (31) reported previously. In our study, 37 cases (35.2%) of KRAS mutations were found in blood tests (22.9% G12D mutation, 8.6% G12V mutation, and 3.8% G12R mutation). Although the positivity rate for *KRAS* mutation detection in patients with advanced pancreatic cancer was even higher, no differences in sensitivity of ctDNA detection for the classification of different stage pancreatic cancer were found, possibly due to the small sample size of I/II stage patients recruited.

Finally, the efficacy of *KRAS* gene mutations on patients' prognosis was investigated. Previous studies indicated that ctDNA was produced by tumor cells prone to metastasis and apoptosis; thus, tumor-originated ctDNA might not be satisfying enough for early diagnosis. However, it is still considered an ideal biomarker for predicting prognosis and relapse (32). *KRAS* gene mutations at codon 12, including G12D (47%), G12V (37%), and G12R (11%), are known as the most common *KRAS* mutations in circulating DNA of patients with pancreatic cancer. However, the results are controversial regarding the types of *KRAS* mutation and survival. It was also demonstrated that patients with the G12V mutation had significantly better overall survival compared with patients with the G12D and G12R mutations (9,33).

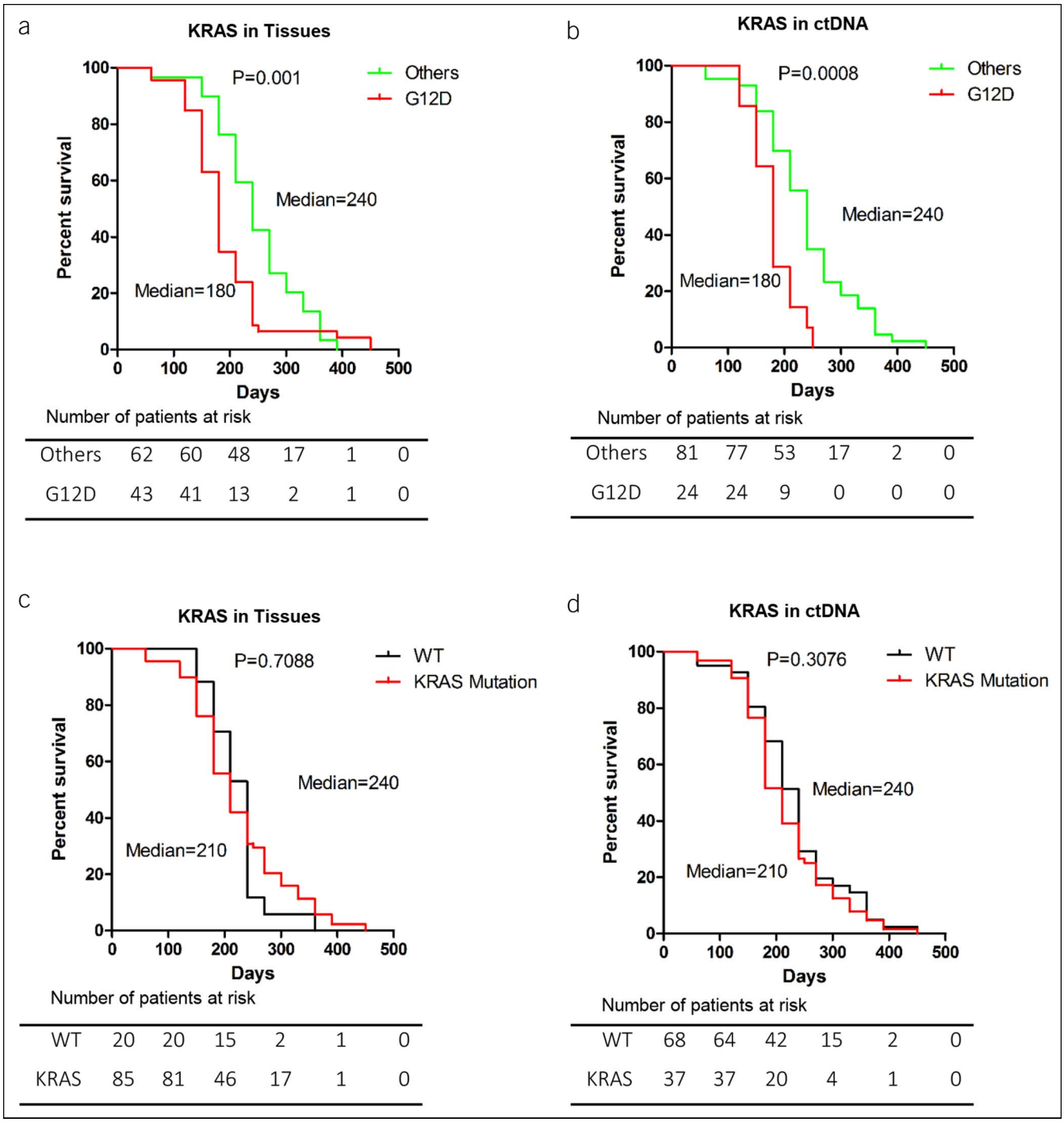

Figure 5. Overall survival of patients. (**a** and **b**) Survival rates of patients with *KRAS* G12D mutations (red line) and other mutations or wild-type (green line). The mutations were detected with either EUS-FNA or ctDNA samples ($P = 0.001$ and $P = 0.0008$, respectively). (**c** and **d**) Survival rates of patients with wild-type *KRAS* (black line) and in those with *KRAS* mutations (red line). There was no significant difference of the MSTs between EUS-FNA and plasma ctDNA groups ($P = 0.7088$ and $P = 0.3076$, respectively). $^{*}P < 0.05$, $^{***}P < 0.001$. ctDNA, circulating tumor DNA; EUS-FNA, endoscopic ultrasound-guided fine-needle aspiration; MST, median survival time; WT, wild type.

By contrast, other studies reported that patients with the G12V mutation possess poorer prognosis than patients with the G12D mutation (34,35). In our study, patients with *KRAS* G12D mutations in pancreatic adenocarcinoma had worse overall survival than those with other mutations. The heterogeneity of tumor characteristics may cause the disparity in these results (36). Multivariate analysis suggested that G12D mutations in both EUS-FNA tissue samples and ctDNA were independent risk factors of poor survival. These results may indicate that ctDNA is produced by the tumor cells prone to metastasis and apoptosis. In addition, the detection of ctDNA may make it possible to detect tumors with heterogeneity. In multivariate analysis, ctDNA

Table 3. Prognostic factors for overall survival by univariate and multivariate analyses in pancreatic cancer (n = 105)

	Univariate analysis HR (95% CI); *P*	Multivariate analysis HR (95% CI); *P*
Age (>65 yr)	1.00 (0.62–1.63); 0.97	NS
Sex (male)	0.84 (0.56–1.27); 0.41	NS
TNM stage (III–IV/I–II)	1.35 (0.74–2.48); 0.33	NS
Location of mass (head, uncinate/body, and tail)	1.28 (0.85–1.93); 0.23	NS
Tumor size (>20 mm)	1.26 (0.69–2.31); 0.45	NS
CEA (>10 ng/mL)	0.64 (0.42–0.99); 0.51	NS
CA19-9 (>37 U/mL)	1.75 (1.15-2.76); 0.04[a]	NS
EUF-FNA *KRAS*	0.85 (0.52–1.39); 0.52	NS
G12D *KRAS*	1.94 (1.12-3.36); <0.0001[b]	1.495 (1.325-1.753); 0.0010[b]
G12V *KRAS*	1.23 (0.80–1.90); 0.35	NS
G12R *KRAS*	0.43 (0.29–0.66); 0.02[a]	NS
ctDNA *KRAS*	0.540 (0.379–1.417); 0.3559	NS
G12D *KRAS*	1.579 (1.383–3.520); 0.0005[b]	1.417 (1.199–2.870); 0.0199[a]
G12V *KRAS*	0.993 (0.393–2.508); 0.9881	NS
G12R *KRAS*	0.296 (0.149–0.589); 0.5276	NS

CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CI, confidence interval; HR, hazard ratio; NS, no significance.
[a]$P<0.05$; [b]$P<0.01$.

KRAS mutations were significantly associated with poor overall survival, indicating that these biomarkers capture unique but complementary prognostic information.

Our study found that adding an analysis of *KRAS* mutations improves the efficacy of EUS-FNA as a tool to diagnose pancreatic cancer over current methods. It also found that *KRAS* mutations are more effectively analyzed in EUS-FNA samples than "liquid biopsy" ctDNA samples and that the presence of these mutations worsens the patients' prognosis.

However, there are several limitations of the study that should be acknowledged. The results we obtained may underestimate the survival benefits of early detection. Most of the patients recruited in this study were with III–IV stage pancreatic cancer. Future studies including a larger number of patients with I–IV stage pancreatic cancer in matched plasma and EUS-FNA samples with a larger quantity of alleles are needed to develop ctDNA analysis an early diagnostic marker in routine clinical application.

CONFLICTS OF INTEREST

Guarantor of the article: Bin Cheng, MD and Jinlin Wang, MD.
Specific author contributions: R.W. and Y.Z.: designed the study and performed data analysis. Y.Z., J.W., and Y.W.: performed acquisition of data. Y.Z.: drafted the manuscript. Q.C.: provided critical revision of the manuscript for important intellectual content. Y.Z., S.X., and Z.Z.: performed technical support. Y.D.: provided pathological verification. B.C. and L.Z.: performed study supervision. All authors have read and approved the manuscript.
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Potential competing interests: None to report.
Availability of data and material: The data sets used and/or analyzed during the current study available from the corresponding author on reasonable request. The EUS-FNA data of patients with pancreatic cancer was upload as Table 1.

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Study Highlights

WHAT IS KNOWN

- ✓ Endoscopic ultrasound-guided fine-needle aspiration is the first-line technique for pancreatic cancer diagnosis, staging, and sampling.
- ✓ The ability of carbohydrate antigen 19-9 to differentiate pancreatic cancer from other benign pancreatic lesions is highly limited.

WHAT IS NEW HERE

- ✓ Digital droplet PCR *KRAS* analysis of fine-needle aspiration samples significantly improved the sensitivity and accuracy of pancreatic cancer diagnosis, superior to the efficacy of carbohydrate antigen 19-9 and noninvasive blood-based circulating tumor DNA.
- ✓ G12D *KRAS* mutations in pancreatic cancer were independently associated with poor overall survival.

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