Diagnostic and prognostic values of KRAS mutations on EUS-FNA specimens and circulating tumor DNA in patients with pancreatic cancer

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DOI: 10.21203/rs.2.20563/v1

SUBJECT AREAS  
Gastroenterology & Hepatology  Oncology

KEYWORDS  
Pancreatic cancer, endoscopic ultrasound, fine needle aspiration, KRAS gene mutations, circulating tumor DNA
Abstract

Background: Early diagnosis is critical in reducing pancreatic cancer mortality. We explore the diagnostic values of detecting KRAS gene mutations and plasma circulating tumor DNA (ctDNA) in patients with primary pancreatic cancer.

Methods: The cohort study is comprised of 149 consecutive patients with pancreatic solid mass underwent Endoscopic ultrasound fine needle aspiration (EUS-FNA) between September, 2014 and May, 2016. KRAS point mutations (G12V, G12D, and G12R) were analyzed by droplet digital PCR (ddPCR) in EUS-guided fine needle aspiration (FNA) histopathology tissue samples and blood samples were tested for plasma ctDNA. The final diagnosis was based on surgical resection pathology or follow up for at least 2 years.

Results: The sensitivity and accuracy of EUS-FNA diagnose was 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. The sensitivity and accuracy of circulating biomarkers combination (ctDNA and CA19-9) were 78.9% and 76.2%, respectively. The median survival time was significantly shorter in patients with G12D mutations (180 days) compared to patients with other mutations (240 days) (long-rank test, P<0.001).

Conclusions: KRAS mutation analysis in tissues significantly improves the sensitivity and accuracy of cyto/histopathological evaluation in EUS-FNA samples. The accuracy of KRAS mutation analysis in EUS-FNA samples was obviously higher than non-invasive blood-based ctDNA KRAS mutation in detecting pancreatic cancer. G12D KRAS mutation in pancreatic cancer were independently associated with poor overall survival.

Background

Pancreatic cancer is the fourth leading cause of cancer-related death in the world, with more than 60,000 estimated deaths per year in China(1). The prognosis has hardly improved over the last 25 years with the median survival remains shorter than 6 months and the 5-year overall survival is only 9% (2). Early cancer diagnosis is critical to the patients' outcome. Currently, pancreatic cancer is routinely diagnosed via endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA), which is the
recommended first-line technique(3). However, EUS-FNA is an invasive procedure, and the sensitivity and accuracy of EUS-FNA can be improved further (4). To potentially improve the diagnostic accuracy, biomarkers molecular analysis of the specimen obtained by EUS-FNA is introduced(5-8). In pancreatic cancer, KRAS mutation is the most commonly acquired mutation with reported rates of 70–95%(9). The KRAS gene encodes p21 RAS protein, which is a small guanosine triphosphatase (GTPase). This point mutation of KRAS, which involved in the process of pancreatic carcinogenesis, uncontrolled proliferation, and invasion of pancreatic cancer cells(10), can be used as a potential biomarker.

When molecular mutation detection in primary pancreatic cancer is limited by the difficulty in obtaining tumor tissues, biomarkers in the circulation provide a noninvasive approach which can be most widely available(11). Recently, mutant circulating tumor DNA (ctDNA) has been explored as a biomarker. The concept underlying this approach, also called “liquid biopsies,” is that cancer cells release DNA from the dying cells into body fluids such as plasma(12, 13). CtDNA is a broadly applicable, sensitive, and specific biomarker that can be used for a variety of clinical and research purposes in patients with multiple different types of cancer(14). The fraction of patients with detectable plasmatic ctDNA as well as its concentration increase with adenocarcinoma stage(15). 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 a higher sensitivity and accuracy in early diagnose of pancreatic cancer. To address these questions, 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 patients. Then we assessed the diagnostic values of KRAS gene mutations in EUS-FNA samples, plasma ctDNA, and prognostic value of KRAS gene mutations in pancreatic cancer (Fig. 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 third study aim was to explore the prognostic value of
KRAS gene mutations in advanced pancreatic adenocarcinoma.

Methods

Patients

The Tongji Medical College of Huazhong University of Science and Technology (HUST) Review Board approved this 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 2016 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; presence of a solid mass lesion was confirmed by at least one imaging modality and was located within pancreas; mass size > 1 cm. Exclusion criteria included: 1) anemia (hemoglobin < 12.4 gm/dL for men and < 11.6 gm/dL for women); 2) pregnancy; 3) coagulopathy (INR > 1.5), thrombocytopenia (platelet count < 50,000/mm3), and acute pancreatitis within the previous 2 weeks, or 4) intervening structures prohibiting needle access.

EUS-FNA technique

All EUS procedures were performed by an experienced endosonographer (B.C.) who had performed more than 1000 procedures. An Olympus linear echoendoscope (GF-UCT 260, GF-UCT 240; Olympus, Tokyo, Japan) and Pentax linear echoendoscope (EG 3870UTK; Pentax, Tokyo, Japan) were used. The material aspirated from all 149 patients was immediately evaluated by the operator to determine if the specimen was sufficient according to results of the macroscopic onsite evaluation (MOSE)(16). 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 the hematoxylin and eosin as well as by immunohistochemical staining if necessary (Figure 2.). The material for KRAS analysis was sent to lab for DNA extraction immediately. Total DNA was extracted from the fresh specimens DNeasy Blood & Tissue Kit ((Qiagen) according to the manufacturer’s instructions.
Extraction of cell-free ctDNA in plasma

The collected blood sample (5 mL) was centrifuged at 3,500 rpm for 15 minutes at 4°C within 3 hours. Then we stored plasma at -80°C for further use. DNA was extracted from plasma with QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer’s instructions. 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; BioRad, Hercules, CA)(17, 18). The DNA was concentrated and distributed among these droplets randomly. The authors detected 3 types of (G12V, G12R and G12D) with PrimePCR products (BioRad) for ddPCR (cat 1863115, 1863112 and 1863113) because these KRAS mutations encompass nearly 90% KRAS mutations in pancreatic cancer(10) (show in Figure 3. A-D). Reactions were performed in 20 mL of reaction system, which consisted of extracted DNA (5 mL), target primer mix (FAM) (1 mL), reference primer/probe mix (HEX) (1 mL), KRAS mutations droplet PCR supermix (10 mL), and distilled water (3 mL). PCR reactions were run on C1000 Touch thermal cycler incubating plate (Bio-Rad) at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds, 10-minute incubation at 98°C. Negative controls without serum ctDNA shown no positive signal. All samples were analyzed in duplicate and variations were set at <5%. The detection rate was set at >0.001%. Schematic and flow diagram of digital PCR in EUS-guided FNA cytology and ctDNA specimens analyses was shown in Figure 1.

Final diagnosis

The final diagnosis was based on pathological examination of 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 non-malignant pancreatic masses. If clinical/imaging follow-up indicated the progression or metastasis of lesion with malignant symptoms, such as weight loss, anemia, or death, the case was considered to be malignant. 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 malignancy or suspicious for malignancy were categorized as positive for malignancy, whereas samples that were considered benign or atypical were categorized as negative for malignancy (16, 19).

**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, 5 patients withdraw, 3 patients lost in follow up. So the authors analyzed 149 patients, including 105 pancreatic cancer patients [age 58.38 ± 11.01 years; male 69/105 (65.71%), 14 cases had TNM staging I-II cancers and 91 cases staging III-IV cancers], and 44 cases with non-malignant pancreatic masses [age 52.66 ± 13.81 years; male 36/44 (81.82%)] (Table 1). These 105 pancreatic cancer cases consisted of 102 pancreatic ductal adenocarcinoma and 3 acinar cell carcinoma. These non-malignant 44 cases consisted of 26 autoimmune pancreatitis (AIP), 10 chronic pancreatitis and 3 pancreatic tuberculosis. The final diagnoses were based on evaluating surgical pathology (n = 29), and clinical courses after 2 years’ follow-up (n = 105). One case with KRAS G12D mutation of chronic pancreatitis was found to be malignant during follow-up.
Diagnostic value of KRAS gene mutations with EUS-FNA specimens in pancreatic adenocarcinoma

Next, authors analyzed the KRAS gene mutations in codons 12 in EUS-FNA samples from primary pancreatic adenocarcinoma (n = 105, Fig. 2, a representative patient) by using digital droplet polymerase chain reaction (ddPCR, Figure.3A-D). The sensitivity, specificity, PPV, NPV, and accuracy of the EUS-FNA alone were, 71.4%, 86.4%, 92.6%, 55.9% and 75.8%, respectively, whereas these values of the EUS-FNA with KRAS mutation analysis were 91.6%, 80.9%, 92.5%, 79.1% and 88.6%, respectively. The sensitivity and accuracy of EUS-FNA diagnose was increased from 71.4–91.6% (P < 0.001) and 75.8–88.6% (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 in pancreatic adenocarcinoma. Detecting KRAS gene mutations improved the diagnose of pancreatic adenocarcinoma with EUS-FNA.

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

Aiming for a non-invasive method for detecting KRAS mutations, the authors evaluated 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, the sensitivity and accuracy of KRAS mutations in EUS-FNA samples were 91.6% and 88.6%, while the respective values of KRAS mutation in ctDNA were 32.8% and 42.7%. In the pancreatic adenocarcinoma group, KRAS gene mutations were found in 88 (83.8%) of 105 cases in primary cancer, while only 37 (35.3%) cases of KRAS mutation were found in ctDNA (p < 0.001, χ2 test, Table S1). The accuracy of non-invasive ctDNA KRAS in detecting pancreatic adenocarcinoma was not as high as EUS-FNS KRAS mutation analysis (P < 0.0001, Table 2 and Table S1). But the sensitivity and accuracy of combined KRAS mutations in plasma ctDNA and CA19-9 were 78.9% and 76.2%, respectively. These results indicated that detecting of circulating biomarkers combination (ctDNA and CA19-9) complements the use of other diagnostic techniques in the diagnosis of pancreatic cancer.

Prognostic value of KRAS gene mutation 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 sample (long-rank test, $P = 0.001$ and $P = 0.0008$, respectively) (Figures. 4A and 4B). In 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 (long-rank test, $P = 0.7088$ and $P = 0.3076$, respectively) (Figures. 4C and 4D).

Furthermore, Univariate analysis demonstrated that both G12D KRAS mutation in EUS-FNA tissue samples (HR, 1.94; 95%CI, 1.12–3.36, $P < 0.0001$) and ctDNA (HR, 1.579; 95%CI, 1.383–3.520, $P < 0.0005$) were significant factors for poor survival. Multivariate analysis demonstrated that 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, $P = 0.0199$) were independently associated with poor overall survival (Table 3). The following factors were analyzed as possible risk factors for survival: age, sex, TNM stage, location of mass, tumor size, CEA, CA19-9, and KRAS mutations (G12D, G12V, and G12R).

In univariate analysis, both baseline CA19-9 and ctDNA KRAS were associated with overall survival (OS), which was expected given the known positive correlation between the two variables.

**Discussion**

The estimated progression of pancreatic adenocarcinoma from stage I to stage IV takes approximately 1 year, although the development of cancer from intraepithelial neoplasia takes up to 10 years (20). Cyto/histopathological evaluation of EUS-FNA samples is one of the most useful methods for early diagnosis of pancreatic masses. However, previous studies have showed that the sensitivity, specificity, and accuracy of EUS-FNA in the diagnosis of pancreatic cancer ranged from 64 to 94%, 71 to 100%, and 78 to 95%, respectively (21–24). Our study demonstrated that KRAS mutation ddPCR analysis significantly improves the sensitivity and accuracy of cyto/histopathological evaluation in EUS-FNA samples. Our study suggested that the accuracy of non-invasive ctDNA KRAS mutation in detecting pancreatic cancer was inferior to that of KRAS mutation analysis in EUS-FNA samples. These results indicated that KRAS mutation in plasma ctDNA, which also termed as “liquid biopsy”, may not complement the use of EUS-FNA in the diagnosis of pancreatic cancer. Our study also demonstrated that KRAS
mutation ddPCR analysis in tissues significantly improves the sensitivity and accuracy of cyto/histopathological evaluation in EUS-FNA samples. Combining KRAS mutation ddPCR analysis and cyto/histopathological in EUS-FNA samples is more valuable than blood test in the diagnose of pancreatic cancer.

Cyto/histopathological evaluation of EUS-FNA samples is one of the most useful methods for early diagnosis of pancreatic masses. Conversely, ctDNA is derived from apoptosis and necrosis of tumor cells, which are characteristic of later stage disease(25). Very few studies have reported the concordance of results between tumor and plasma samples for pancreatic cancer. Therefore, it is difficult to estimate the true diagnostic sensitivity and specificity of these analyses. In this study, the authors demonstrated that the sensitivity, specificity and accuracy of KRAS mutations in EUS-FNA samples were 71.4%, 86.4% and 75.8%, while these values of the KRAS mutation in ctDNA were 32.8%, 85.7% and 42.7%. So the accuracy of KRAS mutation analysis in EUS-FNA samples was obviously higher than non-invasive blood-based ctDNA KRAS mutation in detecting pancreatic cancer.

Detection of early driver mutations in blood samples through “liquid biopsy”(14) does not alternate cyto/histopathological evaluation of EUS-FNA samples. However, detecting of circulating biomarkers combination (ctDNA and CA19-9) is also promising, which may complement the use of other diagnostic techniques in the diagnosis of pancreatic cancer.

In this study, the authors identified KRAS mutations in 35.3% of the ctDNA in plasma samples from patients with pancreatic cancer. This is consistent with the percentage of KRAS-positive ctDNA, specifically 39%(26), 35.3% (27), 32%(22), and 26%(28) reported previously. In this study, 37 (35.3%) cases of KRAS mutation were found in blood test (22.9% G12D mutation, 8.6% G12V mutation and 3.8% G12R mutation). This is in line with other studies were done on matched samples. One is the work of Uemura(22), who detected the KRAS tumor mutation in 35% of plasma samples in stage II to IV. In Nora Brychta’s study(17), KRAS mutations were detected in 35% of plasma samples, G12D mutations were identified in 18% of the analyzed plasma samples, and G12V in 17% of the cases. Given the results above, more recently studies described a lot of efforts to develop new noninvasive blood test to improve the sensitivity, specificity and accuracy of KRAS mutations in ctDNA for the
detection of pancreatic ductal adenocarcinoma. In Joshua D. Cohena study(11), they show combining mutations in circulating tumor DNA (ctDNA) with protein markers, such as CA19-9, CEA, HGF or OPN, can lead to an early detection with improved sensitivity while retaining specificity. K. Allenson’s group show that a higher percentage of patients with localized PDAC exhibited detectable KRAS mutations in exoDNA than ctDNA(27).

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 to the types of KRAS mutation and survival. Some studies demonstrated that patients with the G12V mutation had significantly better overall survival compared with patients with the G12D and G12R mutations(7, 29). While other studies reported that patients with the G12V mutation process poorer prognosis than patients with the G12D mutation(30, 31). In the current study, the authors demonstrated that patients with KRAS G12D mutations in PDAC had worse overall survival than those with other mutations. The controversy may caused by heterogeneity of tumor characteristics(32). Multivariate analysis demonstrated that both G12D mutation in EUS-FNA tissue samples and ctDNA were independent risk factors of poor overall survival. These results may suggest that ctDNA is produced by the tumor cells that are potent to metastasis and apoptosis. In addition, the detection of ctDNA may make it possible to detecting tumor of with heterogeneity. In multivariate analysis, ctDNA KRAS mutations were significantly associated with OS, indicating that these biomarkers capture unique, but complementary prognostic information.

Conclusion
This study demonstrates the feasibility of matched mutational analysis of ctDNA and EUS-FNA samples from the same pancreatic cancer patients. However, there are several limitations of the study that should be acknowledged. The results we obtained may underestimate the survival benefits of early detection. The majority of the patients we studied had 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 larger quantity of alleles, and increased ddPCR or DNA sequencing resolution are needed to develop ctDNA analysis as an early diagnostic marker in routine clinical application.
Abbreviations
Circulating tumor DNA (ctDNA)
Digital droplet polymerase chain reaction (ddPCR)
Endoscopic ultrasound (EUS) Fine needle aspiration (FNA)
V-Ki-ras2 Kirsten rat sarcoma (KRAS)
Pancreatic adenocarcinoma (PDAC)
Computerized tomography (CT)
Magnetic resonance imaging (MRI)
carcinoembryonic antigen (CEA)
carcinoma antigen 19-9 (CA19-9)

Declarations

**Ethics approval and consent to participate:**
The study concerning the clients’ right to privacy. The study was approved by the Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology and was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from the subject, and his study considered declaration of Helsinki as a statement of ethical principles. Informed consent was obtained from all patients.

**Consent for publication**
N/A

**Availability of data and material**
The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.
Competing Interests:

Drs. Ronghua Wang, Jinlin Wang, Yuchong Zhao, Yun Wang, Zhenxiong Zhao, Qian Chen, Yaqi Duan, Bin Cheng have no conflicts of interest or financial ties to disclose.

Funding:

This work was supported by National Natural Science Foundation of China Grants No. 81802418 (to R.W.), No. 81372352 and No. 81172063 (to B.C.).

Authors’ contributions:

R.W. and B.C. designed the study and performed data analysis. R.W, J.W. and Y.W. performed acquisition of data. R.W. drafted the manuscript. Q.C. provided critical revision of the manuscript for important intellectual content. Y. Z. and Z. Z performed technical support. Y. D. provided pathological verification. B.C. performed study supervision. All authors have read and approved the manuscript.

Acknowledgments

We sincerely thank all staff from the Tongji Hospital, Tongji Medical College, HUST for their effort in project execution. We also thank the statistical staffs (Ping Yin, Chang Shu) at the Department of Epidemiology and Biostatistics, School of Public Health, Tongji Medical College, HUST and Hepatic Surgery Centre, Tongji Hospital, Tongji Medical College, HUST.

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Tables

Table 1. Patient characteristics (N =149).

| Characteristics             | Pancreatic Cancer† (n=105) | Non-malignant pancreatic mass‡ (n=44) |
|-----------------------------|----------------------------|---------------------------------------|
| Age, mean ± SD,             | 58.38±11.01                | 52.66±13.81                           |
| Gender                      |                            |                                       |
| Male                        | 696.57%                    | 3681.82%                              |
| Female                      | 3634.29                    | 818.18                                |
| Location of mass            |                            |                                       |
| Head/uncinate               | 7066.67                    | 3886.36                               |
| Body/tail                   | 3533.33                    | 613.64                                |
| CA19-9                      |                            |                                       |
| ≥37 U/mL                    | 7571.43                    | 1022.73                               |
| <37 U/mL                    | 3028.57                    | 3477.27                               |
| CEA                         |                            |                                       |
| ≥10 ng/mL                   | 3230.48                    | 1022.73                               |
| <10 ng/mL                   | 7369.52                    | 3477.27                               |
| Surgery                     |                            |                                       |
| Yes                         | 2422.86                    | 511.36                                |
| No                          | 8177.14                    | 3988.64                               |

†: Pancreatic cancer including 102 ductal adenocarcinoma and 3 acinar cell carcinoma.

‡: Non-malignant pancreatic mass including 26 autoimmune pancreatitis (AIP), 10 chronic pancreatitis, 3 pancreatic tuberculosis.

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 (KRAS) | ctDNA (KRAS) | CA19-9 | Combination |
| Sensitivity   | 71.4%           | 32.8%        | 71.2%  | 78.9%       |
| Specificity   | 86.4%           | 85.7%        | 78.3%  | 77.4%       |
| PPV           | 92.6%           | 90.9%        | 91.2%  | 88.0%       |
| NPV           | 55.9%           | 77.4%        | 46.7%  | 50.2%       |
| Accuracy      | 75.8%           | 42.7%        | 73.0%  | 76.2%       |

ctDNA: circulating tumour DNA;

EUS-FNA: Endoscopic ultrasound-guided fine needle aspiration.

PPV: positive predictive value

NPV: negative predictive value

Combination: the combination of ctDNA and CA199

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

|                          | Univariate analysis | Multivariate analysis |
|--------------------------|---------------------|-----------------------|
|                          | HR (95% CI); P      | HR (95% CI); P        |
| Age>65                   | 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         | 1.28(0.85-1.93);0.23| NS                    |
| (Head,uncinate/Body,tail)|                     |                       |
| Tumor Size≤20mm          | 1.26(0.69-2.31);0.45| NS                    |
| CEA10ng/mL               | 0.64(0.42-0.99);0.51| NS                    |
| CA19-9(37U/mL)           | 1.75(1.15-2.76);0.04*| NS                  |
| EUS-FNA KRAS             | 0.85(0.52-1.39);0.52| NS                    |
| G12D KRAS                | 1.94(1.12-3.36);<0.0001*| 1.495(1.325-1.753);0.0010** |
| G12V KRAS                | 1.23(0.80-1.90);0.35  | NS                    |
| G12R KRAS                | 0.43(0.29-0.66);0.02  | NS                    |
| ctDNA KRAS               | 0.540(0.379-1.417);0.3559| NS                  |
| G12D KRAS                | 1.579(1.383-3.520);0.0005**| 1.417(1.199-2.870);0.0199** |
| G12V KRAS                | 0.993(0.393-2.508);0.9881 | NS                  |
| G12R KRAS                | 0.296(0.149-0.589);0.5276 | NS                  |

CI: confidence interval; HR: hazard ratio; S: No significance.

Figures
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 pancreatic cancer patients using either EUS-guided FNA cytology specimens or ctDNA from blood plasma. KRAS mutations were evaluated for potential clinical utility or as prognostic indicators.
Figure 2

Histopathological features in a representative patient with pancreatic adenocarcinoma. (A)
A representative patient with pancreatic cancer (2.58×3.31cm) in CT (red arrow) and EUS-FNA (yellow arrow), which expressed villin(+), Ki67(+), CDX2(-), CD20(-).
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, G12R) mutation. Signal detected in channel 2, represents DNA positive for KRAS wild type (WT) amplification in which ctDNA mutants was non-detectable.
Overall Survival of patients. (A and B) The survival rates of patients with KRAS G12D mutations (red line) and other mutations or wild-type (green line) were found to be significantly different when the mutations were diagnosed with EUS-FNA and ctDNA samples (P=0.001 and P=0.0008, respectively). (C and D) Survival rates of patients with wild-type KRAS (black line) and those with KRAS mutations (red line). No difference was observed between the median survival times (MSTs) when the mutations were diagnosed in tissues obtained using endoscopic ultrasound guided fine-needle aspiration (EUS-FNA) samples and plasma circulating tumor DNA (ctDNA) (P=0.7088 and P=0.3076, respectively).

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.
supplement Table S1..docx
STROBE_checklist_cohort.doc