Kras Gene Analysis Using Liquid-Based Cytology Specimens Predicts Therapeutic Responses and Prognosis in Patients with Pancreatic Cancer

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Simple Summary: New therapeutic strategies are needed to improve the prognosis of pancreatic ductal adenocarcinoma (PDAC) and developing biomarkers that can guide individualized treatment decisions is an important part of these strategies. In this study, we found that unresectable PDAC patients harboring wild-type Kras had significantly longer progression-free survival (PFS) and overall survival (OS) than those harboring mutant Kras after undergoing first-line gemcitabine and nab-paclitaxel (GA) therapy and that wild-type Kras was a significant predictor of longer PFS and OS. This is the first report suggesting that Kras gene analysis has the potential to predict therapeutic responses to GA and the prognosis of unresectable PDAC.

Abstract: Background: Although several molecular analyses have shown that the Kras gene status is related to long-term survival of patients with pancreatic ductal adenocarcinoma (PDAC), the results remain controversial. Here, we examined the Kras gene status in a cohort of unresectable PDAC patients who underwent first-line therapy with gemcitabine and nab-paclitaxel (GA) and assessed differences in chemotherapy responses and survival. Methods: Patients with a histological diagnosis of PDAC (based on EUS-guided fine-needle aspiration) from 2017 to 2019 were enrolled. Tumor genomic DNA was extracted from residual liquid-based cytology specimens and Kras mutations were assessed using the quenching probe method. The relationships between the Kras status and progression-free survival (PFS) and overall survival (OS) were assessed. Results: Of the 110 patients analyzed, 15 had wild-type Kras. Those with the wild-type gene showed significantly longer PFS and OS than those with mutant Kras (6.9/5.3 months (p = 0.044) vs. 19.9/11.8 months (p = 0.037), respectively). Multivariate analyses identified wild-type Kras as a significant independent factor associated with longer PFS and OS (HR = 0.53 (p = 0.045) and HR = 0.35 (p = 0.007), respectively). Conclusions: The analysis of the Kras gene status could be used to predict therapeutic responses to GA and prognosis in unresectable PDAC patients.

Keywords: Kras; pancreatic ductal adenocarcinoma; pancreatic cancer; gemcitabine and nab-paclitaxel; EUS-FNA; liquid-based cytology
1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer-related death in the United States [1] and is projected to become the second leading cause of cancer-related death worldwide in the next decade [2]. At the time of diagnosis, most patients have locally advanced or metastatic disease and <20% of patients have curatively operable disease [3]. Chemotherapy is the mainstay of treatment for locally advanced and metastatic disease and novel chemotherapy regimens such as FOLFIRINOX and gemcitabine plus nab-paclitaxel (GA) have improved prognosis for these patients [4,5]. Although progress has been made, long-term survival rates remain low, with <10% of patients remaining alive 5 years after diagnosis [6]. New treatment strategies are needed to significantly improve survival. One of the most promising tools is to establish biomarkers that can guide individualized treatment decisions.

Liquid-based cytology (LBC) is a technique commonly used (alongside conventional smear (CS) preparation) for the analysis of specimens obtained by endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA). LBC is a thin-layer slide preparation technique developed to overcome the disadvantages of CS, such as cell clouding and blood contamination [7–9]. Moreover, since most of the collected cells can be used as LBC samples, a genomic analysis can be performed easily using the LBC samples that remain after cytological diagnosis [10]. Sekita-Hatakeyama et al. described the usefulness of residual LBC specimens for \textit{Kras} mutation analysis in pancreatic cancer [11].

Mutations in the \textit{Kras} oncogene are driver mutations; as such, they are an initiating event in PDAC. These mutations are present in >90% of PanIN lesions and in >90% of PDAC cases [12]. A meta-analysis of the data from 17 retrospective studies reported that the detection of \textit{Kras} mutations might be a useful poor prognostic factor for PDAC [13]. However, this meta-analysis included data from patients in different clinical settings and treatments. Moreover, a multicenter prospective study by Hass et al. reported no association between \textit{Kras} mutations and prognosis [14]. Thus, it is still controversial whether \textit{Kras} mutations contribute to the poor prognosis of PDAC. In addition, the association between \textit{Kras} mutations and prognosis needs to be evaluated in cases with standardized background factors (such as clinical setting and treatment).

Here, we analyzed \textit{Kras} mutations using residual LBC specimens from EUS-FNA of patients with unresectable PDAC who underwent first-line GA therapy. We then examined differences in response to chemotherapy and survival.

2. Materials and Methods

2.1. Study Design

The protocol for this single-center cohort study was approved by the Wakayama Medical University Ethical Review Board (no. 3253). The study was conducted in accordance with the tenets of the Declaration of Helsinki.

The primary endpoints were PFS and OS of patients with unresectable PDAC who underwent first-line GA chemotherapy. These endpoints were evaluated according to differences in the \textit{Kras} mutation status in residual LBC specimens from EUS-FNA. The factors predictive of PFS and OS were also analyzed.

2.2. Patients

The study included LBC specimens from 278 consecutive patients who underwent EUS-FNA at Wakayama Medical University Hospital between February 2017 and December 2019. Written informed consent was obtained from all research participants.

Inclusion criteria were (1) age ≥ 20 years, (2) a pathological diagnosis of PDAC by EUS-FNA, (3) unresectable locally advanced or metastatic PDAC and (4) first-line treatment chemotherapy using a GA regimen. Exclusion criteria were (1) LBC cytology class I or class II, (2) unsuccessful \textit{Kras} mutation measurement and (3) less than one full course of first-line GA chemotherapy.
2.3. EUS-FNA Procedure and Specimen Processing

Patients underwent EUS-FNA using a GF-UCT260 linear echoendoscope (Olympus Medical, Tokyo, Japan) connected to an ultrasound scanning system (ARIETTA 850; Fujifilm, Tokyo, Japan). The pancreatic lesion was punctured using a 22/25G aspiration needle (Expect™ or Acquire™; Boston Scientific, Natick, MA, USA; or EZ Shot3™; Olympus Medical, Tokyo, Japan) under real-time ultrasound guidance. The stylet was withdrawn and aspirated (10 cc negative pressure) using the attached syringe and the aspiration needle was moved back and forth 20 times within the lesion before being withdrawn from the echoendoscope. Finally, the aspirated material was pushed out into a preservative liquid (ThinPrep CytoLyt Solution; Hologic, Marlborough, MA, USA) by reinsertion of the stylet.

The aspirated material was separated for histological evaluation, cytological evaluation and Kras mutation analysis. The solid materials were fixed in 10% formalin, embedded in paraffin and sliced thinly for additional immunostaining as required. The liquid material was treated using the ThinPrep® method according to the manufacturer’s recommendations and then evaluated immediately by Papanicolaou staining. The residual LBC specimens were stored at 4 °C until DNA extraction.

2.4. DNA Extraction and Kras Mutation Analysis

Residual LBC samples were centrifuged at 2000 rpm for 10 min. Genomic DNA was purified from the sediment using Maxwell RSC FFPE Kit-PKK Custom (Promega, Madison, WI, USA). The amount of DNA quantity was measured using a Quantus™ Fluorometer (Promega) and a QuantiFluor ONE dsDNA System (Promega). Gene mutations were examined for Kras codons (codons 12, 13, 59 and 61) and for the corresponding wild-type Kras using the fully automated genotyping system i-densy (IS-5320; ARKRAY, Tokyo, Japan) [15,16].

2.5. Definitions

Tumor diameter on contrast-enhanced computed tomography (CE-CT) was measured before chemotherapy. Responses to chemotherapy were classified according to the RECIST guidelines (ver. 1.1) as follows: complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). The tumor reduction rate was calculated from the CE-CT as follows: (tumor size before chemotherapy minus tumor size after chemotherapy)/tumor size before chemotherapy. PR was defined as a 30% decrease in the longest diameter and PD as a 20% increase in the longest diameter. Observations were performed once every 2–3 months during the course of treatment.

2.6. Chemotherapy

Almost all patients received nab-paclitaxel (125 mg/m²) and gemcitabine (1000 mg/m²) as first-line chemotherapy. In some cases, the dose was reduced according to the general condition of the patient, such as age and performance status. Patients were followed carefully after initial treatment (by imaging and monitoring of tumor markers). Patients were treated with GA therapy until PD was observed. Patients with PD were offered a second-line chemotherapy regimen or the best supportive care. The start date of the follow-up was set as the date of initiation of first-line chemotherapy for PDAC. The end date of the follow-up was set as the final follow-up in May 2021 or the time of patient death.

2.7. Statistical Methods

The primary endpoints were PFS and OS of patients with unresectable PDAC who underwent first-line GA chemotherapy. With respect to background data, the significance of the differences in continuous data was assessed using non-paired Student’s t-tests as a reference. The chi-squared test was used to analyze categorical data. PFS and OS were measured from the first day of chemotherapy to the date of PD and death, respectively. The survival curves for PFS and OS were estimated using the Kaplan–Meier method.
Univariate and multivariate analyses using a Cox proportional hazard model were performed to identify variables significantly associated with PFS and OS. *p*-values < 0.05 were considered statistically significant. The statistical analyses were performed using JMP Pro ver. 14 (SAS Institute, Inc., Cary, NC, USA).

3. Results

Figure 1 shows the study flow chart. During the study period, the Kras gene statuses of 278 consecutive patients were examined. Of the patients assessed for enrollment, 168 were excluded and 110 were analyzed. The demographic and baseline characteristics of these patients are summarized in Table 1. There were 95 patients with mutant *Kras* and 15 with wild-type *Kras*. A total of 69 patients (63%) received second-line treatment, such as TS-1 (*n* = 51), FOLFIRINOX (*n* = 11), or nanoliposomal irinotecan plus fluorouracil and folinic acid (*n* = 7). The other patients were provided with the best supportive care. Patient factors such as sex, age, ECOG performance status, lesion size, disease status, CA19-9 expression, amount of extracted DNA and receipt of second-line chemotherapy were not significantly different between the two groups. However, patients with wild-type *Kras* had more head lesions than patients with mutant *Kras* (*p* = 0.012), although there was no significant difference in tumor size (Table 2).

![Figure 1. Study flow chart.](image-url)

| Table 1. Clinical characteristics of study patients. |
|--------------------------------------------------|
|                                                        |
| **n = 110**                                            |
| Sex, male/female                                      | 47/73 |
| Patient age, y, mean (range)                          | 67.8 (44–82) |
| Performance status 0/1                                | 104/6 |
| Lesion size, mm, mean (range)                         | 28.8 (9.8–72) |
| Location of lesion head/body or tail                  | 54/56 |
Table 1. Cont.

| n = 110          |
|------------------|
| Disease status   |
| local/ metastatic| 30/80 |
| CA19-9, mg/mL    |
| <37/ ≥37         |
| 21/89            |
| Kras mutation status |
| mutant/wild type |
| 95/15            |
| Amount of extracted DNA, ng/µL, mean (range) |
| 28.2 (1.6–217.0) |
| Second line chemotherapy * |
| absent/present   |
| 38/69            |

*Three patients were still on first-line chemotherapy.

Table 2. Characteristics of patients according to the two groups.

|                          | Kras, Mutant (n = 95) | Kras, Wild Type (n = 15) | p-Value |
|--------------------------|------------------------|--------------------------|---------|
| Sex, male/female, n (%)  | 55/42 (57.9/42.1)      | 9/6 (60/40)              | 1.000   |
| Patient age, y, mean     | 68.4                   | 64.3                     | 0.102   |
| Performance status 0/1, n (%) | 88/9 (92.6/7.4)   | 14/1 (93.3/6.7)          | 1.000   |
| Lesion size, mm, mean    | 29.1                   | 27.1                     | 0.524   |
| Location of lesion head/body or tail, n (%) | 42/53 (44.2/55.8) | 12/3 (80/20)              | 0.012   |
| Disease status local/metastatic, n (%) | 24/71 (25.3/74.7) | 6/9 (40/60)              | 0.348   |
| CA19-9, mg/mL <37/ ≥37, n (%) | 19/78 (20/80) | 4/11 (26.7/73.3)          | 0.480   |
| Amount of extracted DNA, ng/µL, mean | 29.4 | 20.5 | 0.384 |
| Second line chemotherapy absent/present, n (%) | 32/61 (52.5/47.5) | 6/8 (40/60)              | 0.560   |

CA19-9, carbohydrate antigen 19-9.

The comparisons of response to first-line chemotherapy with GEM and nab-PTX were assessed between the wild-type Kras and the mutant Kras groups. According to RECIST v1.1, in the wild-type Kras group, 3 (20%) showed a PR, 10 (66.7%) showed SD and 2 (13.3%) showed PD, and in the mutant Kras group, 15 patients (15.8%) showed a PR, 40 (42.1%) showed SD and 40 (42.1%) showed PD. The rate of objective response was observed in 3 patients (20%) in the wild-type Kras group and 15 patients (15.8%) in the mutant Kras group (p = 0.701; Table 3). The rate of disease control was observed in 13 patients (86.7%) in the wild-type Kras group and 55 patients (57.9%) in the mutant Kras group (p = 0.044; Table 3). There was a significant difference in the rate of disease control between two groups.

The PFS and OS of patients, classified according to the Kras status, are shown in Figures 2 and 3. The median PFS in the wild-type Kras and the mutant Kras groups were 6.9 months (95% CI = 3.3–11.2) and 5.3 months (95% CI = 2.3–6.2), respectively, and the median OS was 19.9 months (95% CI = 11.1–NA) and 11.8 months (95% CI = 7.3–18.4), respectively. Both PFS and OS were significantly longer in the wild-type Kras group (p = 0.044 and p = 0.037, respectively; log-rank test; see Figures 2 and 3).
Table 3. Response to first-line chemotherapy with GEM and nab-PTX.

| t | kras, mutant (n = 95) | kras, wild type (n = 15) | p-value |
|---|-----------------------|--------------------------|---------|
| Response, no. (%) | Complete response | 0 (0) | 0 (0) | |
| Partial response | 15 (15.8) | 3 (20) | |
| Stable disease | 40 (42.1) | 10 (66.7) | |
| Progressive disease | 40 (42.1) | 2 (13.3) | |
| Rate of objective response *, no. (%) | 15 (15.8) | 3 (20) | 0.701 |
| Rate of disease control **, no. (%) | 55 (57.9) | 13 (86.7) | 0.044 |

* The rate of objective response was defined as the percentage of patients who had a complete response or partial response. ** The rate of disease control was defined as the percentage of patients who had a complete response, partial response, or stable disease.

Factors associated with PFS and OS were identified using the Cox proportional hazard model. Only wild-type kras was associated significantly with longer PFS in the univariate analysis (HR = 0.56, 95% CI = 0.31–0.99; p = 0.049) and multivariate analysis (HR = 0.53, 95% CI = 0.28–0.99; p = 0.045) (Table 4). Local advanced disease (HR = 0.60, 95% CI = 0.37–0.96; p = 0.026); kras, wild-type (HR = 0.50, 95% CI = 0.26–0.97; p = 0.026); and second-line chemotherapy (HR = 0.44, 95% CI = 0.29–0.68; p < 0.001) were significantly associated with longer OS in the univariate analysis (Table 5). The multivariate analysis identified local advanced disease (HR = 0.57, 95% CI = 0.36–0.92; p = 0.048); kras, wild-type (HR = 0.35, 95% CI = 0.16–0.74; p = 0.007); and second-line chemotherapy (HR = 0.20, 95% CI = 0.20–0.50; p < 0.001) as significant independent factors associated with longer OS (Table 5).
Figure 3. Overall survival according to \textit{Kras} status after first-line chemotherapy with GEM and nab-PTX. The median OS in the wild-type \textit{Kras} and the mutant \textit{Kras} group was 19.9 and 11.8 months, respectively. OS was significantly longer in the wild-type \textit{Kras} group than in the mutant \textit{Kras} group ($p = 0.037$).

Table 4. Univariate and multivariate analyses of prognostic factors with progression-free survival.

|                          | Univariate HR (95% CI) | p-Value | Multivariate HR (95% CI) | p-Value |
|--------------------------|------------------------|---------|--------------------------|---------|
| Sex                      | Female/male            | 0.99 (0.67–1.45) | 0.992 | 0.94 (0.60–1.46) | 0.769 |
| Patient age, y           | >70/≦70                | 0.99 (0.67–1.45) | 0.946 | 1.04 (0.68–1.60) | 0.842 |
| Performance status       | 1/0                    | 1.46 (0.64–3.34) | 0.368 | 1.60 (0.65–3.96) | 0.309 |
| Lesion size, mm          | >20/≦20                | 1.19 (0.77–1.85) | 0.424 | 1.12 (0.69–1.81) | 0.639 |
| Location of lesion       | Body or tail/head      | 1.09 (0.74–1.59) | 0.665 | 1.03 (0.69–1.81) | 0.886 |
| Disease status           | Local/metastatic       | 0.83 (0.54–1.28) | 0.399 | 0.98 (0.61–1.57) | 0.924 |
| \textit{Kras} status     | Wild type/mutant       | 0.56 (0.31–0.99) | 0.049 | 0.53 (0.28–0.99) | 0.045 |
| CA19-9, mg/mL            | ≧37/<37                | 0.95 (0.59–1.55) | 0.850 | 0.89 (0.53–1.49) | 0.658 |

CA19-9, carbohydrate antigen 19-9; HR, hazard ratio; 95% CI, 95% confidence interval.

Table 5. Univariate and multivariate analyses of prognostic factors associated with overall survival.

|                          | Univariate HR (95% CI) | p-Value | Multivariate HR (95% CI) | p-Value |
|--------------------------|------------------------|---------|--------------------------|---------|
| Sex                      | Female/male            | 0.87 (0.58–1.31) | 0.560 | 1.10 (0.69–1.77) | 0.462 |
| Patient age, y           | >70/≦70                | 0.93 (0.62–1.40) | 0.735 | 0.97 (0.61–1.52) | 0.853 |
| Performance status       | 1/0                    | 1.66 (0.72–3.81) | 0.230 | 1.33 (0.54–3.25) | 0.491 |
| Lesion size, mm          | >20/≦20                | 1.26 (0.82–1.83) | 0.313 | 1.10 (0.67–1.81) | 0.860 |
| Location of lesion       | Body or tail/head      | 0.82 (0.55–1.83) | 0.323 | 1.16 (0.75–1.79) | 0.424 |
| Disease status           | Local/metastatic       | 0.60 (0.37–0.96) | 0.026 | 0.57 (0.36–0.92) | 0.048 |
| \textit{Kras} status     | Wild type/mutant       | 0.50 (0.26–0.97) | 0.026 | 0.35 (0.16–0.74) | 0.007 |
| CA19-9, mg/mL            | ≧37/<37                | 1.02 (0.60–1.74) | 0.932 | 0.62 (0.34–1.10) | 0.104 |
| Second line chemotherapy  | Present/absent         | 0.44 (0.29–0.68) | <0.001 | 0.20 (0.20–0.50) | <0.001 |

CA19-9, carbohydrate antigen 19-9; HR, hazard ratio; 95% CI, 95% confidence interval.
4. Discussion

In this study, we analyzed Kras mutations in residual LBC specimens of EUS-FNA samples from patients with unresectable PDAC who underwent first-line GA therapy. We then assessed the differences in response to chemotherapy and survival. We found that patients with wild-type Kras had significantly longer PFS and OS than those with mutant Kras, suggesting that wild-type Kras could be used as a predictor of longer PFS and OS. These findings suggest that Kras gene analysis can predict therapeutic responses to GA, as well as the prognosis of patients with PDAC.

Previous studies attempted to determine the role of Kras as a prognostic biomarker for the clinical outcome of PDAC. A meta-analysis that pooled data from 17 retrospective studies reported that Kras mutations might be a useful prognostic factor for PDAC [13]. However, this meta-analysis had two major problems. First, some cohorts included fewer cases with the Kras mutation (60–70% of cases) than other studies, which usually report 90% prevalence of Kras mutations, suggesting that the gene analyses used in these studies were of low quality. Second, the analysis included cases from various clinical settings with different treatments, such as surgery, chemotherapy and the best supportive care, in which the patients’ backgrounds were also not consistent. Haas et al. conducted a multicenter prospective study of unresectable PDAC and reported no significant difference in OS between patients with wild-type Kras and mutant Kras (9.9 vs. 8.3 months, \( p = 0.70 \), respectively) PDAC [14]. By contrast, an observational study of unresectable PDAC by Windon et al. noted that the OS of wild-type Kras patients was higher than that of mutant Kras patients (736 vs. 420 days, \( p = 0.026 \), respectively) [17]. This discrepancy may be due to differences in first-line chemotherapy regimens among patients. Therefore, in the present study, first-line chemotherapy was standardized to reduce bias related to patient background.

Although the mechanism for the longer PFS and OS of wild-type Kras patients compared with mutant Kras patients after first-line GA therapy was not elucidated, we hypothesized as follows. First, mutations in Kras cause constitutive activation of RAS and perpetuate downstream signaling pathways involved in cellular proliferation, migration, apoptosis and cytoskeletal remodeling, independent of growth factor receptor activation [18]. Sustained activation of RAS signaling had been reported as a major cause of gemcitabine resistance [19] and inhibiting Kras signaling suppresses migration and invasion in a gemcitabine-resistant cell line [20,21]. Therefore, Kras gene mutation may contribute to acquiring resistance and poor response to gemcitabine. Second, Schlitter et al. performed a histological analysis of surgical resection specimens and found that patients with colloid, medullary, or papillary carcinoma survived significantly longer than patients with conventional PDAC (\( p = 0.04 \)) [22]. These findings suggest that wild-type Kras PDACs may be less likely to grow and spread rapidly than Kras-mutant PDACs. Third, on the basis of genome profile analyses, it was report that, compared with mutant Kras PDACs, wild-type Kras PDACs have one of the following three characteristics: (1) presence of an activated-MAPK in the absence of a Kras mutation, such as deleterious genetic changes in BRAF, GNAS, EGFR, ERBB2, MET, ERBB3, MAP2K4, FGFR1, NTRK1 and ERBB4; (2) presence of microsatellite instability/defective DNA mismatch repair; or (3) presence of kinase-fusion genes, such as FGFR2, RAF, ALK, RET, MET, NTRK, ERBB4 and FGFR3 [23–25]. These gene mutations may contribute to a better response to GA, although we did not analyze these gene mutations in this study and the mechanism is unknown. In the future, it will be necessary to analyze not only the Kras mutation status but also the whole genome mutational landscape including these mutations to examine the response to GA and OS.

As for Kras gene analysis, formalin-fixed paraffin-embedded (FFPE) tissues or frozen tissue samples are commonly used for the genomic analysis of PDAC [26]. However, the amount of specimen obtained by EUS-FNA of a pancreatic lesion can be minimal; therefore, it is not always possible to obtain enough DNA for a multi-gene analysis. By contrast, the advantage of the molecular analysis of LBC specimens is that it can be performed using only liquid specimens, meaning that the remaining specimens used for cytology can be used [27].
Several reports described Kras mutation analysis using LBC specimens from lesions in other organs, especially the lungs. Zhao et al. reported that they successfully extracted DNA from LBC samples collected from EUS-FNA tumor tissues of lung cancer patients and performed real-time PCR in all cases [28]. In our study, we successfully detected Kras mutations in 98.6% (274/278 cases) of residual LBC specimens, suggesting the usefulness of the genomic analysis using LBC specimens. Furthermore, even if a sufficient amount of tissue can be obtained by EUS-FNA, it needs to be stored for future MSI testing or multi-gene panel analysis. Therefore, it is advantageous to use LBC specimens rather than FFPE for Kras single-gene analysis at the time of diagnosis [29,30].

The study has several limitations. First, it is a single-center retrospective study enrolling a relatively small number of patients to standardize patient backgrounds. In the future, a large-scale, multicenter study will be necessary. Second, the Tm analysis method using a quenching probe and i-densyTM may enable a complete and fully automated analysis of polymorphisms in extracted DNA within about 60 min; further, the detection of Kras mutations using this method is better than the direct sequencing method (indeed, it is equivalent to the Scorpion-ARMS method, which has relatively high sensitivity) [31]. However, the major problem with this method is that it cannot detect Kras mutation subtypes. Currently, therapeutic drugs targeting Kras G12C are being developed and it will be necessary to detect Kras subtypes in the future [32]. Third, the presence and type of second chemotherapy and disease status (local or metastatic) were not standardized in our study. The second chemotherapy and disease status are considered to be very important for OS. Although the Kras status, presence of second chemotherapy and disease status were found to be independent factors for OS, further studies are needed to investigate the relationship between the Kras status and OS in patients who have received the same chemotherapy regimens and have the same disease status.

5. Conclusions

In conclusion, patients with PDAC harboring wild-type Kras had significantly longer PFS and OS than those with mutant Kras. In addition, the multivariate analysis revealed that wild-type Kras was a significant predictor of longer PFS and OS of patients with unresectable PDAC who underwent first-line GA therapy. These findings suggest that Kras gene analysis can predict therapeutic responses to GA, as well as the prognosis of patients with PDAC.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated during the study will be available from the corresponding author on reasonable request after termination of data collection.

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References

1. Siegel, R.L.; Miller, K.D.; Fuchs, H.E.; Jemal, A. Cancer Statistics, 2021. CA Cancer J. Clin. 2021, 71, 7–33. [CrossRef]

2. Rahib, L.; Smith, B.D.; Aizenberg, R.; Rosenzweig, A.B.; Fleshman, J.M.; Matrisian, L.M. Projecting cancer incidence and deaths to 2030: The unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res. 2014, 74, 2913–2921. [CrossRef] [PubMed]

3. Luchini, C.; Capelli, P.; Scarpa, A. Pancreatic Ductal Adenocarcinoma and Its Variants. Surg. Pathol. Clin. 2016, 9, 547–560. [CrossRef] [PubMed]

4. Von Hoff, D.D.; Ervin, T.; Arena, F.P.; Chiorean, E.G.; Infante, J.; Moore, M.; Seay, T.; Tjulandin, S.A.; Ma, W.W.; Saleh, M.N.; et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N. Engl. J. Med. 2013, 369, 1691–1703. [CrossRef]

5. Conroy, T.; Desseigne, F.; Ychou, M.; Bouche, O.; Guimbaud, R.; Becouarn, Y.; Adenis, A.; Raoul, J.L.; Gourgou-Bourgade, S.; de la Fouchardiere, C.; et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N. Engl. J. Med. 2011, 364, 1817–1825. [CrossRef] [PubMed]

6. Siegel, R.L.; Miller, K.D.; Jemal, A. Cancer statistics, 2020. CA Cancer J. Clin. 2020, 70, 7–30. [CrossRef]

7. Linder, J. Recent advances in thin-layer cytology. Diagn. Cytopathol. 1998, 18, 24–32. [CrossRef]

8. Chun, J.W.; Lee, K.; Lee, S.H.; Kim, H.; You, M.S.; Hwang, Y.J.; Paik, W.H.; Ryu, J.K.; Kim, Y.T. Comparison of liquid-based cytology with conventional smear cytology for EUS-guided FNA of solid pancreatic masses: A prospective randomized noninferiority study. Gastrointest. Endosc. 2020, 91, 837–846. [CrossRef]

9. Akagi, K.; Arai, Y. Tm analysis method using a quenching probe is a simple and rapid way to simultaneously detect KRAS and BRAF mutations. Rinsho. Byori. 2011, 59, 757–762. [PubMed]

10. Suzuki, S.; Komori, M.; Hirai, M.; Ureshino, N.; Kimura, S. Development of a novel, fully-automated genotyping system: Principle and applications. Sensors 2012, 12, 16614–16627. [CrossRef] [PubMed]

11. Windon, A.L.; Loaiza-Bonilla, A.; Jensen, C.E.; Randall, M.; Morrissette, J.J.D.; Shroff, S.G. A KRAS wild type mutational status confers a survival advantage in pancreatic ductal adenocarcinoma. J. Gastrointest. Oncol. 2018, 9, 1–10. [CrossRef]

12. Sekita-Hatakeyama, Y.; Nishikawa, T.; Hori, Y.; Horiguchi, S.; Saragai, Y.; Takada, S.; Yabe, S.; Muro, S.; et al. Evaluation of Local Recurrence of Pancreatic Cancer by KRAS Mutation Analysis Using Washes from Endoscopic Ultrasound-Guided Fine-Needle Aspiration. Dig. Dis. Sci. 2020, 65, 2907–2913. [CrossRef]

13. Tao, L.Y.; Zhang, L.F.; Xiu, D.R.; Yuan, C.H.; Ma, Z.L.; Jiang, B. Prognostic significance of K-ras mutations in pancreatic cancer: A meta-analysis. World J. Surg. Oncol. 2016, 14, 146. [CrossRef]

14. Haas, M.; Ormanns, S.; Baechmann, S.; Remold, A.; Kruger, S.; Westphalen, C.B.; Siveke, J.T.; Wenzel, P.; Schlitter, A.M.; Esposito, I.; et al. Extended Ras analysis and correlation with overall survival in advanced pancreatic cancer. Br. J. Cancer 2017, 116, 1462–1469. [CrossRef]

15. Akagi, K.; Arai, Y. Tm analysis method using a quenching probe is a simple and rapid way to simultaneously detect KRAS and BRAF mutations. Rinsho. Byori. 2011, 59, 757–762. [PubMed]

16. Suzuki, S.; Komori, M.; Hirai, M.; Ureshino, N.; Kimura, S. Development of a novel, fully-automated genotyping system: Principle and applications. Sensors 2012, 12, 16614–16627. [CrossRef] [PubMed]

17. Windon, A.L.; Loaiza-Bonilla, A.; Jensen, C.E.; Randall, M.; Morrissette, J.J.D.; Shroff, S.G. A KRAS wild type mutational status confers a survival advantage in pancreatic ductal adenocarcinoma. J. Gastrointest. Oncol. 2018, 9, 1–10. [CrossRef]

18. Hingorani, S.R.; Tuveson, D.A. Ras redux: Rethinking how and where Ras acts. Curr. Opin. Genet. Dev. 2003, 13, 6–13. [CrossRef]

19. Shimizu, K.; Nishiyama, T.; Hori, Y. Gemcitabine Enhances Kras-MEK-Induced Matrix Metalloproteinase-10 Expression Via Histone Acetylation in Gemcitabine-Resistant Pancreatic Tumor-initiating Cells. Cancer Lett. 2017, 46, 268–275. [CrossRef] [PubMed]

20. Kang, Y.W.; Lee, J.E.; Jung, K.H.; Son, M.K.; Shin, S.M.; Kim, S.J.; Fang, Z.; Yan, H.H.; Park, J.H.; Han, B.; et al. KRAS targeting antibody synergizes anti-cancer activity of gemcitabine against pancreatic cancer. Cancer Lett. 2018, 438, 174–186. [CrossRef]

21. Ryu, W.J.; Han, G.; Lee, S.H.; Choi, K.Y. Suppression of Wnt/β-catenin and RAS/ERK pathways provides a therapeutic strategy for gemcitabine-resistant pancreatic cancer. Biochem. Biophys. Res. Commun. 2021, 549, 40–46. [CrossRef] [PubMed]

22. Schlitter, A.M.; Segler, A.; Steiger, K.; Michalski, C.W.; Jäger, C.; Konukiewitz, B.; Pfarr, N.; Endris, V.; Bettstetter, M.; Kong, B.; et al. Molecular, morphological and survival analysis of 177 resected pancreatic ductal adenocarcinomas (PDACs): Identification of prognostic subtypes. Sci. Rep. 2017, 7, 41064. [CrossRef]

23. Singh, A.D.; George, B.; Greenbowe, J.R.; Chung, J.; Suh, J.; Maitra, A.; Klemmner, S.J.; Hendifar, A.; Milind, J.M.; Golan, T.; et al. Real-Time Targeted Genome Profile Analysis of Pancreatic Ductal Adenocarcinomas Identifies Genetic Alterations That Might Be Targeted with Existing Drugs or Used as Biomarkers. Gastroenterology 2019, 156, 2242–2253. [CrossRef]

24. Luchini, C.; Paolino, G.; Mattiolo, P.; Piredda, M.L.; Cavaliere, A.; Gaule, M.; Melisi, D.; Salvia, R.; Malleo, G.; Shin, J.I.; et al. KRAS wild-type pancreatic ductal adenocarcinoma: Molecular pathology and therapeutic opportunities. J. Exp. Clin. Cancer Res. 2020, 39, 227. [CrossRef]

25. Luchini, C.; Brosens, L.A.A.; Wood, L.D.; Chatterjee, D.; Shin, J.I.; Sciammarella, C.; Fadone, G.; Malleo, G.; Salvia, R.; Kryklyva, V.; et al. Comprehensive characterisation of pancreatic ductal adenocarcinoma with microsatellite instability: Histology, molecular pathology and clinical implications. Gut 2021, 70, 148–156. [CrossRef] [PubMed]
26. Yokose, T.; Kitago, M.; Matsuda, S.; Sasaki, Y.; Masugi, Y.; Nakamura, Y.; Shinoda, M.; Yagi, H.; Abe, Y.; Oshima, G.; et al. Combination of KRAS and SMAD4 mutations in formalin-fixed paraffin-embedded tissues as a biomarker for pancreatic cancer. *Cancer Sci.* 2020, **111**, 2174–2182. [CrossRef]

27. Kwon, H.; Kim, W.G.; Eszlinger, M.; Paschke, R.; Song, D.E.; Kim, M.; Park, S.; Jeon, M.J.; Kim, T.Y.; Shong, Y.K.; et al. Molecular Diagnosis Using Residual Liquid-Based Cytology Materials for Patients with Nondiagnostic or Indeterminate Thyroid Nodules. *Endocrinol. Metab.* 2016, **31**, 586–591. [CrossRef]

28. Zhao, H.; Qiu, T.; Guo, H.; Ying, J.; Li, J.; Zhang, Z. Detection of EGFR and KRAS gene mutations using suspension liquid-based cytology specimens in metastatic lung adenocarcinoma. *Oncotarget* 2017, **8**, 106685–106692. [CrossRef]

29. Park, J.K.; Lee, J.H.; Noh, D.H.; Park, J.K.; Lee, K.T.; Lee, J.K.; Lee, K.H.; Jang, K.T.; Cho, J. Factors of Endoscopic Ultrasound-Guided Tissue Acquisition for Successful Next-Generation Sequencing in Pancreatic Ductal Adenocarcinoma. *Gut Liver* 2020, **14**, 387–394. [CrossRef]

30. Sugimoto, M.; Irie, H.; Takagi, T.; Suzuki, R.; Konno, N.; Asama, H.; Sato, Y.; Nakamura, J.; Takasumi, M.; Hashimoto, M.; et al. Efficacy of EUS-guided FNB using a Franseen needle for tissue acquisition and microsatellite instability evaluation in unresectable pancreatic lesions. *BMC Cancer* 2020, **20**, 1094. [CrossRef]

31. Suzuki, S.; Matsusaka, S.; Hirai, M.; Shibata, H.; Takagi, K.; Mizunuma, N.; Hatake, K. A novel approach to detect KRAS/BRAF mutation for colon cancer: Highly sensitive simultaneous detection of mutations and simple pre-treatment without DNA extraction. *Int. J. Oncol.* 2015, **47**, 97–105. [CrossRef] [PubMed]

32. Indini, A.; Rijavec, E.; Ghidini, M.; Cortellini, A.; Grossi, F. Targeting KRAS in Solid Tumors: Current Challenges and Future Opportunities of Novel KRAS Inhibitors. *Pharmaceutics* 2021, **13**, 653. [CrossRef] [PubMed]