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KRAS G12D Mutation Subtype Is A Prognostic Factor for Advanced Pancreatic Adenocarcinoma

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OBJECTIVES: There is no molecular biomarker available in the clinical practice to assess the prognosis of advanced pancreatic carcinoma. This multicenter prospective study aimed to investigate the role of *KRAS* mutation subtypes within the primary tumor to determine the prognosis of advanced pancreatic cancer.

METHODS: The exon-2 *KRAS* mutation status was tested on endoscopic ultrasound-guided fine-needle aspiration biopsy material (primary tumor; restriction fragment-length polymorphism plus sequencing and TaqMan allelic discrimination) of patients with proven locally advanced and/or metastatic pancreatic ductal carcinoma. We used the Kaplan–Meier method, log-rank test, and Cox's model to evaluate the impact of *KRAS* status on the overall survival (OS), adjusting for age, stage of disease, clinical performance status, CA 19-9 levels, and treatment.

RESULTS: A total of 219 patients (men: 116; mean age: 67 $\pm$ 9.4 years) were included: 147 harbored a codon-12 *KRAS* mutation (G12D: 73; G12V: 53; G12R: 21) and 72 had a wild-type *KRAS*. There was no difference in the OS between patients with a mutant *KRAS* (8 months; 95% confidence interval (95% CI): 8.7–12.3) and the wild-type (9 months; 95% CI: 8.7–12.8; hazard ratio (HR): 1.03; $P = 0.82$). However, the patients with a G12D mutation had a significantly shorter OS (6 months; 95% CI: 6.4–9.7) compared with the other patients (OS: 9 months; 95% CI: 10–13; HR: 1.47; $P = 0.003$; i.e., wild type: 9 months, G12V: 9 months, G12R: 14 months). Similar results were observed in the subgroup of 162 patients who received chemotherapy (HR: 1.66; $P = 0.0013$; G12D ($n = 49$): 8 months, wild type ($n = 56$): 10 months, G12V ($n = 38$): 10 months, G12R ($n = 19$): 14 months). Multivariate analyses identified *KRAS* G12D as an independent predictor for worse prognosis within the entire series (HR: 1.44; $P = 0.01$) and in the subgroup of patients that received chemotherapy (HR: 1.84; $P = 0.02$).

CONCLUSIONS: The *KRAS* G12D mutation subtype is an independent prognostic marker for advanced pancreatic ductal carcinoma. Codon and amino-acid-specific mutations of *KRAS* should be considered when evaluating the prognoses as well as in trials testing drugs that target RAS and downstream RAS pathways.

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INTRODUCTION

Pancreatic cancer remains one of the most deadly types of cancer: the 5-year survival rate after diagnosis is $<3.5\%$.[1] The sole potentially curative treatment is surgical resection, but it is applicable in no more than 15% of patients. Single-agent gemcitabine, FOLFIRINOX, and combinations of nab–paclitaxel–gemcitabine, although they do not dramatically improve survival beyond 11 months, have demonstrated significant clinical benefits and have become the standard chemotherapy for advanced and metastatic pancreatic ductal adenocarcinoma (PDAC).[1] Among the prognostic factors that have been identified in cases of advanced non-resectable PDAC, only standard and common clinical or biological items have been retained, such as age, metastasis, clinical performance status, CA 19-9 serum level, and treatment with chemotherapy. No molecular biomarker is available at present that can help or influence the management of PDAC patients.

Molecular characterization of PDAC had revealed that *INK4a/ARF*, *TP53*, and *DPC4/Smad4* suppressor pathways are genetically inactivated in the majority of cases, whereas driver oncogenic *KRAS* is activated.[2,3] The activating point mutation of the *KRAS* oncogene on codon-12 remains the major event. It is present in 70–95% of PDAC cases and 71% of pancreatic cancer specimens within the COSMICS database harbor *KRAS*.[4] The single-nucleotide mutation on codon-12 of exon-2 induces replacement of the GGT sequence (encoding for glycine) by the GAT sequence (aspartic acid–G12D–c35 G>A), GTT (valine–G12V–c35 G>T), CGT (arginine–G12R–C34 G>C), or GCT (alanine–G12A–c35 G>C). A point mutation can also occur, but less frequently, on codon-13 (G13D) or -61 (Q61L or Q61H).[3–5]

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The *KRAS* gene encodes for the protein P21 RAS, which is a small GTPase that acts as a molecular switch by coupling cell-membrane growth-factor receptors to intracellular signaling pathways and transcription factors to control various cellular processes. The point mutation of *KRAS* impairs intrinsic GTPase activity of RAS and prevents GTPase-activating proteins to promote conversion of GTP (active) to GDP (inactive). P21 RAS is thus permanently bound to GTP and activates downstream signaling pathways, such as PI3K/AKT/mTOR, RAF, or MEK/ERK (independently of upstream growth-factor receptor activation).[5] Following this activation, nuclear transcription factors are also activated with the stimulation of cell proliferation, transformation, adhesion, and survival.[1,5]

Several groups have investigated whether the presence of a *KRAS* mutation can influence the prognosis of PDAC. In these studies, the number of *KRAS* mutations found in samples (biopsies or resected specimens) varies between 41 and 75%.[6–16] In addition, series have mixed both resected and non-resectable (locally advanced and/or metastatic) PDAC patients. In some studies, ampullary carcinomas or non-resectable PDAC and a former resected tumor with subsequent recurrence have been included. Despite these discrepancies, the presence of a *KRAS* mutation seems to negatively affect survival, regardless of performing curative surgery or not. As already observed for lung carcinoma, it has been pointed out that the *KRAS* mutational subtype may also negatively influence prognosis *per se*.[17,18]

We conducted a multicenter prospective study to assess the prognostic role of *KRAS* mutation subtypes within the primary tumor and in a homogenous cohort of patients with advanced PDAC. The objective was to assess whether *KRAS* mutation subtypes influence the patient's overall survival (OS) or not.

METHODS

Patients. A total of 219 patients with histologically proven metastatic or locally advanced PDAC were included between April 2005 and April 2013 in four French referral centers (Clichy, Marseille, Montpellier, and Toulouse). All the patients were referred for an endoscopic ultrasound-guided fine-needle aspiration biopsy (EUS-FNA) based on the results of previous imaging techniques (abdominal ultrasound, EUS, computed tomography scan, or magnetic resonance cholangiopancreatography), which suggested a pancreatic cancer. The confirmation of PDAC was obtained after analysis of the FNA sampled from the primary tumor. EUS-FNA was repeated in cases where there was an initial non-contributive sample until a diagnosis was confirmed.

The following patients were excluded: those previously treated with chemotherapy, those who had a first-line pancreatic resection or had received a neoadjuvant treatment with chemotherapy and/or radiotherapy, those with a pancreatic tumor that differed from PDAC, or where the histology was only obtained from a metastatic site. The protocol was approved by the regional ethical committee (CCPPRB Midi-Pyrénées II January 2005: protocol N°03 042 02, including external audit of the data). Informed written and signed consent was obtained from all the patients.

EUS-FNA and samples for *KRAS* analyses. EUS was performed under intravenous propofol anesthesia, as previously described,[19] using a curved linear array EUS type FG-36 UA Pentax (Argenteuil, France) or an UCT140T Olympus (Rungis, France) connected to Hitachi or Aloka ultrasound device, respectively. EUS-FNA was performed using a Wilson–Cook EUSN1-22-gauge needle (Limerick, Ireland). A minimum of two needle passes were required until sufficient tissue material was collected. A core-biopsy sample of pancreatic tissue was transferred into either Dubosq-Brazil, Formaline, or Cytolyte media using a needle stylet: these samples were used for further cytological and histological diagnoses, including immunohistochemistry. Once the core biopsies were retrieved, the stylet was removed and the cellular material remaining in the needle catheter was air flushed with a sterile 20-ml syringe and placed into a sterile 1-ml Eppendorf and immediately frozen to −20 °C until DNA extraction.

***KRAS* mutation analyses.** *KRAS* assays were centralized in one center. EUS-FNA samples were then centrifuged for 10 min at 8,000 r.p.m. DNA was extracted from the pellets using a QIAamp DNA micro-kit (QIAGEN, Les Ulis, France). Nucleic acid quantization was done using a Nanovue spectrophotometer (GE Healthcare, Buckinghamshire, UK). To identify *KRAS* codon-12 mutations (c.34G>C p.G12R; c.35G>A p.G12D; c.35G>T p.G12V), we performed a two-step nested PCR amplification, followed by restriction fragment-length polymorphism analysis, as previously described.[19] DNA sequencing using a Big Dye Terminator v3.1 Kit in an automatic ABI 3100 sequencer (Applied Biosystems, Foster City, CA) allowed us to verify and identify mutations of a first or second nucleotide in codon-12 and possible mutations in codon-13 in cases where there was a wild-type codon-12. Since January 2010, *KRAS* codon-12 mutations have been assessed using a mutation detection assay based on custom TaqMan MGB dual probes, as previously described.[20] During a 6-month period, both the techniques (restriction fragment-length polymorphism plus sequencing and TaqMan MGB allelic discrimination) were conducted in parallel and provided identical results.[20] The investigators who performed the *KRAS* assays were blinded to the pathological diagnoses.

Data recorded, follow-up, and treatments. Baseline data included symptoms and a medical history, World Health Organization (WHO) performance status, standard laboratory assessments, serum CA 19-9 level, radiological staging, and American Society of Anesthesiology score. After histological confirmation of PDAC from the primary tumor and *KRAS* mutation analysis, the patients were routinely managed in the four referral centers according to the standard of care and national guidelines. When the treatment was decided upon, the multidisciplinary staff did not know the *KRAS* status of each patient to ensure that this did not influence the therapeutic decision, which was based on symptoms, performance status, stage of disease, and comorbidities, which followed the French national guidelines for the management of digestive cancers (www.tncd.org/). The patients were then allocated to receive best supportive care

Table 1 Main characteristics of the patients

	Total	*KRAS* wild type	*KRAS* mutated	*P* value	G12D	G12V	G12R	*P* value
Sex								
Male	116	41	75	0.49	42	24	9	0.13
Female	103	31	72		31	29	12	
Mean Age (Median)	67 ± 9.4 (68)	65 ± 9.2 (66)	68 ± 9.4 (69)	**0.024**	68 ± 9.6 (69)	69 ± 8.3 (70)	64 ± 10.5 (65)	0.06
WHO PS score								
≤1	123	45	78	0.19	32	29	15	0.07
>1	96	27	69		41	24	6	
CA 19-9[a]								
N	25	4	22	0.15	8	6 27	8	0.12
> N	123	38	84		43		14	
Tumor stage								
Locally	103	28	75	0.11	30	31	15	0.052
Metastasis	116	44	72		43	22	8	
Treatments								
Chemotherapy	162	56	106	0.41	49	38	19	0.1
BSC	57	16	41		24	15	2	

BSC, best supportive care; WHO PS score, World Health Organization performance status score.
[a]Baseline CA 19-9 level was assessed only on 149 patients (no evaluation in the 70 remaining patients because of obstructive jaundice, a Lewis blood group, or missing data).
The bold entries highlights the significant data.

or to receive first-line chemotherapy. Subsequent chemotherapy lines could be proposed depending on the clinical course of the disease. All the patients had at least one monthly clinical evaluation and bi-monthly imaging assessment. The data recorded were date and cause of death, new clinical events, and date of last follow-up. The OS was defined as the interval of time between inclusion (date of the cytopathological diagnosis of PDAC combined with the *KRAS* assay) and death.

Statistical analyses. Qualitative and quantitative data were analyzed using Student's *t*-test, the χ^2 test, or Fisher's exact test, as appropriate, with GraphPad-Instat 3.1a software (GraphPad Software, La Jolla, CA). Actuarial survival analyses, according to the Kaplan–Meier model, were calculated using R (version 3.2.3 The R Foundation Statistical Computing), GraphPad Prism (version 6.1), and Stata (version 14) softwares. Comparison of survival rates between the subgroups was performed using the log-rank test. Univariate and multivariate analyses of prognostic factors were carried out using the log-rank test, Wald's test, and Cox's model. A *P* value of <0.05 was considered statistically significant.

RESULTS

Patients' characteristics. The characteristics according to *KRAS* mutation status in 219 patients with advanced PDAC (head of the pancreas: 58%) are shown in **Table 1**. Among the 219 patients, 147 (67%) had a mutant codon-12 *KRAS* mutation. Among these, the most frequent subtype was c.35G>A (G12D; 73 patients; 49.5%), followed by c.35G>T (G12V; 53 patients; 36%) and c.34G>C (G12R; 21 patients; 14.5%). Among the first 140 patients included, no codon-13 *KRAS* mutation was found. For this reason, codon-13 was not subsequently included in the TaqMan allelic discrimination assay until the end of the inclusion period. There were no significant differences between the patients with wild-type and mutated *KRAS* in terms of sex distribution, WHO performance status score, CA-19-9 level, tumor stage, or treatments received. The patients in the *KRAS* mutated subgroup were significantly older (68 ± 9.4 years). Among patients with different *KRAS* mutation subtypes, there were no significant differences in terms of sex distribution, age, WHO performance status, CA 19-9 serum levels, tumor status, or treatments (**Table 1**).

Of the total 162 patients, 119 (73.5%) received first-line chemotherapy with gemcitabine alone, 14 (8.5%) received gemcitabine plus oxaliplatin, 21 (13%) received FOLFIRINOX, and 8 (5%) received another treatment. Second- and third-line chemotherapies were given to, respectively, 39.5% and 10.5% of these patients (gemcitabine, 5-flurouracil plus oxaliplatin or cisplatin, or an oral fluoropyrimidine). The remaining 57 patients received only best supportive care because of their poor performance status, advanced age, or their comorbidities.

Survival according to KRAS mutation subtypes and others prognostic factors in all patients with advanced PDAC. All the patients were followed up until death. From the univariate analyses, two clinical factors appeared to have a significant negative influence on prognosis: a WHO performance status score of ≥1 and having no chemotherapy (i.e., best supportive care; **Table 2**). There was no statistical difference in terms of OS between patients regarding wild-type ($n=72$) or mutated ($n=147$) *KRAS* status (cumulating all codon-12 mutation subtypes). However, the presence of G12D was significantly associated with a worse prognosis when compared with other *KRAS* statuses (i.e., cumulating *KRAS* wild types: G12R and G12V), and to other mutations subtypes (G12V or G12R; **Table 3**). Representative plots according to the Kaplan–Meier test are shown on **Figure 1** and **Supplementary Figure 1** online. Multivariate analyses

Table 2 Analysis of prognostic factors and codon-12 *KRAS* status in 219 patients with locally advanced and/or metastatic pancreatic adenocarcinoma

Variable (*n*)	Overall survival (months): median (95% CI)	Univariate analyses (log-rank test) HR (95% CI); *P* value	Multivariate analyses (Cox's model): HR (95% CI); *P* value
Age			
≤65 years (76)	9 (9–12.8)	1	1
>65 years (143)	8 (8.4–11.3)	1.12 (0.85–1.48); 0.09	1.01 (0.99–1.02); 0.14
WHO PS score			
≤1 (123)	11 (11.3–14.2)	1	1
>1 (96)	5 (5.7– 8.8)	**2.2 (1.68–2.87); <0.0001**	**1.61(1.14–2.26); 0.006**
CA 19-9			
≤*n* (25)	6.5 (5–13)	1	—
>*n* (123)	8 (9–12.3)	1.2 (0.79–2.0); 0.34	
Metastasis			
No (103)	9 (9.5–13.2)	1	1
Yes (116)	7 (8–10.8)	1.24 (0.96–1.64); 0.10	1.08 (0.78–1.51); 0.62
Chemotherapy			
No (57)	4 (4.2– 8.9)	1	1
Yes (162)	10 (10.4– 12.9)	**0.53 (0.28–0.59); <0.0001**	0.82 (0.56–1.19); 0.3
KRAS mutation			
No (72)	9 (8.7–12.8)	1	1
Yes (147)	8 (8.7–12.3)	1.03 (0.78–1.37); 0.82	0.82 (0.48–1.41); 0.48
G12D KRAS mutation			
No (146)	9 (10–12.9)	1	1
Yes (73)	6 (6.4–9.7)	**1.47 (1.19–2.20); 0.0036**	**1.44 (1.00–2.08); 0.01**
G12V KRAS mutation			
No (166)	9 (8.7–14.6)	1	1
Yes (53)	8 (8.7–11)	1.2 (0.91–1.65); 0.19	1.00 (0.56–1.78); 0.99
G12R KRAS mutation			
No (198)	8 (8.5–11)	1	1
Yes (21)	14 (10–18)	0.68 (0.46–1.03); 0.08	1.08 (0.64–1.81); 0.75

95% CI, 95% Confidence interval; HR, hazard ratio; WHO PS score, World Health Organization performance status score.
The bold entries highlights the significant data.

Table 3 Analysis of the prognostic impact of codon-12 *KRAS* mutation subtypes on 219 patients with locally advanced and/or metastatic pancreatic adenocarcinoma

Comparative analyses	Log-rank test: HR (95% CI)	*P* value
G12D vs. WT	1.34 (1.02–1.97)	0.05
G12D vs. G12V	1.43 (1.07–2.15)	**0.03**
G12D vs. G12R	1.81 (1.22–2.86)	**0.008**
G12V vs. G12R	1.2 (0.75–1.96)	0.43
G12V vs. wild type	0.88 (0.61–1.2)	0.47
G12R vs. wild type	0.71 (0.45–0.95)	0.14

95% CI, 95% Confidence interval; HR, hazard ratio; WT, wild type.
The bold entries highlights the significant data.

showed that a WHO performance status score of ≥1 and a G12D *KRAS* mutation were negative prognostic factors (**Table 2**).

Survival according to KRAS mutation subtype and other prognostic factors in patients with advanced PDAC that also received chemotherapy. Univariate analyses revealed that a performance status score of ≥1 and a G12D *KRAS* mutation were negative prognostic factors in the subgroup of 162 patients who received chemotherapy (**Table 4**). There was no statistical difference in terms of OS between patients with wild-type *KRAS* and those with a *KRAS* mutation (cumulating all codon-12 mutation subtypes). Conversely, the presence of G12D was significantly associated with a worse prognosis when compared with any other *KRAS* status (**Table 5**). Considering the homogeneous subgroup of 119 patients that all received first-line gemcitabine, G12D remained significantly associated with a worse prognosis: G12D (median survival 8 months) vs. wild type (median survival 9.5 months—hazard ratio (HR): 2; $P=0.0047$), G12D vs. G12V (median survival 10 months—HR:1.8; $P=0.041$), G12D vs. G12R (median survival 13.5 months—HR: 2.07; $P=0.028$; **Supplementary Table 1**). Representative plots, according to the Kaplan–Meier method, are shown on **Figure 2**. Multivariate analyses showed that a WHO performance status score of ≥1 and a G12D *KRAS* mutation were still negative prognostic factors in patients who received chemotherapy (**Table 4**).

DISCUSSION

In this multicentric study, we prospectively investigated the role of *KRAS* mutations on the prognosis of a homogenous group of patients with advanced PDAC. We observed that the presence of the *KRAS* codon-12 mutation subtype, G12D, in the primary tumor was a negative prognostic factor for reduced

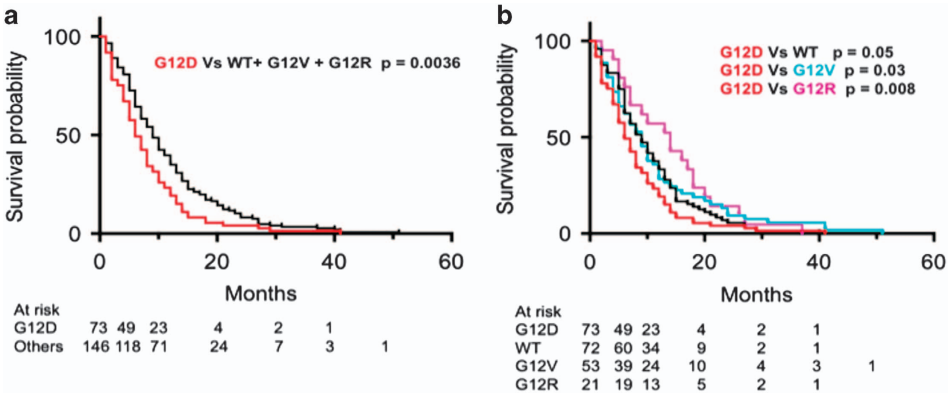

Figure 1 Kaplan–Meier analysis of overall survival times depending on the presence of a G12D *KRAS* codon-12 mutation or not in 219 patients with locally advanced and/or metastatic pancreatic adenocarcinoma. (**a**) Median overall survival in months (95% CI) *KRAS* G12D: 6 (6.4–9.7); *KRAS* wild type+*KRAS* G12V+*KRAS* G12R: 9 (10–12.9). (**b**) Median overall survival in months (95% CI) *KRAS* G12D: 6 (6.4–9.7); *KRAS* G12V: 8 (8.7–11); *KRAS* G12R: 14 (10–18); *KRAS* wild type: 9 (8.7–12.8). 95% CI, 95% confidence interval; WT, wild type.

Table 4 Analysis of prognostic factors and codon-12 *KRAS* status in 162 patients with locally advanced and/or metastatic pancreatic adenocarcinoma treated with chemotherapy

Variable (*n*)	Overall survival (months): median (95% CI)	Univariate analyses (log-rank test) HR (95% CI); *P* value	Multivariate analyses, Cox's model; HR (95% CI); *P* value
Age			
≤65 years (66)	10 (9.9–13.8)	1	1
>65 years (92)	9.5 (9.7–13.1)	1.05 (0.77–1.41); 0.72	1.01 (0.99–1.02); 0.25
WHO PS score			
≤1 (122)	10.5 (11.2–14.2)	1	**1**
>1 (40)	6 (6.4–10.3)	**1.76 (1.42–3.31); 0.0007**	**1.71 (1.18–2.49); 0.004**
CA 19-9			
≤*n* (15)	9 (6.8–14.2)	1	—
>*n* (109)	9 (9.8–13.2)	1.2 (0.48–1.5); 0.59	
Metastasis			
No (75)	10 (10.3–14.6)	1	1
Yes (87)	9 (9.4–12.6)	1.14 (0.85–1.58); 0.36	1.08 (0.78–1.51); 0.25
KRAS mutation			
No (56)	10 (9.8–13.9)	1	1
Yes (106)	9.5 (9.9–13.1)	1.02 (0.74–1.41); 0.88	1.08 (0.78–1.51); 0.61
G12D KRAS mutation			
No (113)	11 (11–14.5)	1	**1**
Yes (49)	8 (7.2–10.4)	**1.66 (1.33–2.85); 0.0013**	**1.79 (1.14–2.79); 0.01**
G12V KRAS mutation			
No (124)	9.5 (9.9–12.4)	1	1
Yes (38)	10 (9.8–16.5)	0.80 (055–1.11); 0.20	1.02 (0.59–1.85); 0.91
G12R KRAS mutation			
No (143)	9 (9.8–12.5)	1	1
Yes (19)	14 (10.8–19.3)	0.68 (0.45–1.04); 0.09	1.04 (0.59–1.83); 0.87

95% CI, 95% Confidence interval; HR, hazard ratio; WHO PS score, World Health Organization performance status score.
The bold entries highlights the significant data.

OS. In addition, a *KRAS* G12D mutation remained a negative prognostic factor in the subgroup of patients who received chemotherapy. Taken together, we conclude that for patients with locally advanced and/or metastatic PDAC, a G12D *KRAS* mutation within a primary tumor is an independent prognostic factor that results in significantly decreased OS, including within the subgroup that received chemotherapy.

Several groups have investigated whether the presence or not of a *KRAS* mutation influences the prognosis of PDAC.[6–16] However, populations of patients were often not homogeneous as they included either patients with resected PDAC or non-resectable PDAC, or a former resected tumor with subsequent recurrence,[7,10] or an ampullary carcinoma.[8,11] It is noteworthy that in studies that included resected carcinoma

only, the tumor was located in the head part in 80 to 90% of the cases.[6,8,11,16] Conversely, in series that included advanced and/or metastatic carcinoma the primary tumor is located in the head part in only 45 to 60% of cases (58% in the present study).[7,9,10,15] A better resectability of tumors of the head of the pancreas might explain this discrepancy. Four of the six studies that focused on resected PDAC patients reported that a codon-12 *KRAS* mutation had a negative influence on the prognosis.[6,8,14,16] Six studies reported this same analysis for unresectable PDAC (i.e., locally advanced and metastatic PDAC), of which four concluded that a *KRAS* mutation was a negative prognostic factor.[7,9,10,13–15] One of these studies was retrospective, and was conducted by Ogura *et al.*[15] who pointed out that the association of codon-12 G12D and G12R mutations negatively influenced prognosis *per se*. In our study, we did not find a significant difference in terms of OS between patients with wild-type *KRAS* or a mutated *KRAS* (including all mutation subtypes). However, our study shows, for the first time, that a *KRAS* G12D mutation was an independent prognostic factor after both uni- and multivariate analyses.

We have applied TaqMan quantitative PCR method to FNA materials because previous work performed on formalin-fixed paraffin-embedded tissue has demonstrated that quantitative PCR methods (including allelic discrimination using the TaqMan assay) when compared with sequencing were equally efficient at detecting hot-spot *KRAS* mutations.[20–22] In addition, the selectivity of sequencing using the *KRAS* assay in tumor tissues was between 15 and 30%, whereas mutation-specific allelic discrimination was between 1 and 5% in colon cancer tissues.[21,22] Beside a possible better selectivity, quantitative PCR was cheaper and faster than RFLP plus sequencing.[19,20] Finally, the total amount of DNA used for TaqMan PCR is lower (50 vs. 150 ng).[19,20] It is thus possible and more easy to perform other molecular analysis such as *BRAF* mutations. We thus investigated the V600E *BRAF* mutation in wild-type *KRAS* samples. Finally, none of these samples were mutated for *BRAF* (data not shown).

In patients with lung adenocarcinoma (non-small cell lung carcinoma), the prognostic role of *KRAS* mutation subtype has also been demonstrated. G12V and G12C mutations could both positively or negatively influence progression-free survival depending on the stage of disease and the treatment given.[17,18,23,24] This effect was not observed with other *KRAS* mutation subtypes.

Similarly, in non-small cell lung carcinoma, it has been shown, using proteomic and modelization studies, that the RAS protein is differentially coupled to downstream signaling pathways depending on the type of mutation.[17] The mutation subtype G12D is associated with phosphorylation and coupling of the PI3K/AKT and MEK cascades, whereas mutation G12V (as well as G12C) preferentially activates the RAF/Ral pathway and decreases phosphorylation of AKT.[17,23,25] These results should be reproduced in PDAC, as PI3K signaling is well known to be implicated in the progression, metastatic power, and chemoresistance of PDAC.[26,27] This could be one of the explanations for the independent implication for the G12D mutation subtype to be associated with worse prognosis for the patients in our study.

Although two clinical factors significantly influenced the prognosis either negatively (performance status score >1) or favorably (administration of chemotherapy) in univariate analyses, only performance status score remained significant in the multivariate analyses. However, both performance and administration of chemotherapy appeared to be collinear variables, with an inverted odds ratio and a similar significance

Table 5 Analysis of prognostic impact of codon-12 *KRAS* mutation subtypes on 162 patients with locally advanced and/or metastatic pancreatic adenocarcinoma treated with chemotherapy

Comparative analysis	Log-rank test: HR (95% CI)	*P* value
G12D vs. WT	1.49 (1.10–2.45)	**0.02**
G12D vs. G12V	1.61 (1.59–2.61)	**0.015**
G12D vs. G12R	2.03 (1.39–3.63)	**0.002**
G12V vs. G12R	1.15 (0.71–2.01)	0.45
G12V vs. wild-type	0.87 (0.57–1.29)	0.49
G12R vs. wild-type	0.73 (0.44–1.17)	0.22

95% CI, 95% Confidence interval; HR, hazard ratio.
The bold entries highlights the significant data.

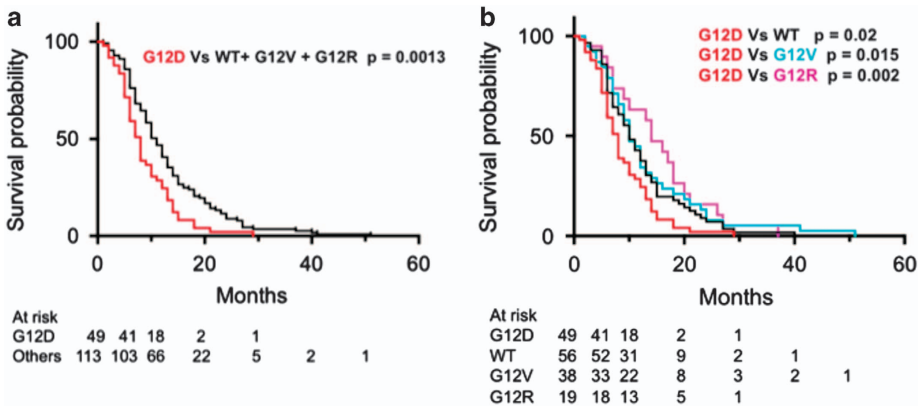

Figure 2 Kaplan–Meier analysis of overall survival times depending on the presence of a G12D *KRAS* codon-12 mutation or not in 162 patients with locally advanced and/or metastatic pancreatic adenocarcinoma treated with chemotherapy. (**a**) Median overall survival in months (95% CI) *KRAS* G12D: 8 (7.2–10.4); *KRAS* wild type+*KRAS* G12V +*KRAS* G12R: 11 (11–14.5). (**b**) Median overall survival in months (95% CI) *KRAS* G12D: 8 (7.2–10.4); *KRAS* G12V: 10 (9.8–16.5); *KRAS* G12R: 14 (10.8–19.3); *KRAS* wild type: 10 (9.8–13.9). 95% CI, 95% confidence interval; WT, wild type.

after univariate analyses. Moreover, 99% of patients with a PS status of ≤ 1 received chemotherapy. Finally, when removing the performance status from Cox's model, the administration of chemotherapy became statistically significant in the multivariate analyses.

From a clinical point of view, we chose to study the subgroup of 162 patients who received chemotherapy (119 gemcitabine, 14 GEMOX protocol, 21 FOLFIRINOX protocol, and 8 miscellaneous). We have shown, for the first time that the G12D mutation is also a negative prognostic factor in PDAC patients treated by chemotherapy, regardless of type. The relationship between a G12D mutation and the worse OS in patients treated with chemotherapy is unknown, but the fact that this mutation could preferentially activate the PI3K/AKT and MEK pathways could explain a chemoresistant tumor phenotype compared with other mutations. These observations require broader validation at both experimental and clinical levels.

It is well known that the *KRAS* mutation status dramatically influences the response to anti-EGFR therapy in metastatic colorectal cancer.[28] In case of PDAC, several studies have shown that activation of the *KRAS* intracellular pathways by mutations did not influence the results from gemcitabine-based treatments.[13,29] From our study, *KRAS* G12D is an independent prognosis factor in the subgroup of patients treated with the single agent gemcitabine. There is thus an opportunity to proposed a "tailored treatment" such as molecules targeting mutated p21 RAS or downstream RAS activated pathways. In other terms, we believe that the *KRAS* mutation subtype should be considered as they may influence therapeutic decisions and specific protocols targeting RAS pathways.

One approach to neutralizing the RAS protein is to vaccinate using RAS peptides that bear different mutations at the amino-acid level. However, up until now, no clinical benefit has been obtained using a vaccination strategy,[30,31] although new protocols have been designed with new vaccination peptides, which are currently being clinically evaluated.[32]

Downstream of RAS posttranslational and membrane-bound processing, the other key targets are the signaling pathways activated by mutated P21KRAS. One of these is MEK, and several MEK inhibitors have been developed. Recently, two small-molecular MEK inhibitors (selumetinib and trametinib) have been tested in phase I and II studies in PDAC patients, either alone or compared with capecitabine, or combined with gemcitabine.[27,33,34] Some patients have responded to the MEK inhibitors, but further studies are needed to assess whether or not these inhibitors, in combination with FOLFIRINOX, are beneficial, and so merit further phase III studies. Apart from the MEK inhibitors, numerous RAF, PI3K, AKT, or mTOR inhibitors are also currently being tested.[35,36] However, some limitations and issues in the application of MEK inhibitors and other compounds have emerged, such as toxicity (general, ocular, skin) and acquired resistance.[27,33,34] However, some cases of partial tumor response have been noted in early phase I trials. Among the downstream RAS effectors, guanine exchange factors RAL (RAL A and RAL B GTPases) are also implicated in the transformation and invasion of pancreatic cancer cells.[37,38] Upon activation, RAL GTPase regulates numerous biological processes such as autophagy, cytokine activation, endocytosis, filopodia formation, membrane trafficking, and transcription. Abnormal regulation of these biological processes regulated by RAL can lead to proliferation, resisting cell death, cell invasion, and metastasis.[38] Simultaneous targeting of RAS or downstream effector such as RAL A and RAL B may provide a novel therapeutic approach of PDAC. For example, in non-small cell lung carcinoma, it has been suggested that RAL GTPase inhibition is an important treatment strategy for tumors that harbor RAS G12C or G12V mutations, which are commonly found in this cancer.[17,23–25]

In conclusion, the *KRAS* G12D mutant seems to be an independent prognostic marker in patients with unresectable pancreatic cancer, particularly in those who are eligible for chemotherapy. These findings provide further support for testing for the *KRAS* mutation subtypes in advanced PDAC to evaluate prognosis, and for clinical trials to test therapies on the downstream RAS pathways, such as PI3K or MEK, which could be preferentially activated by G12D RAS.

CONFLICT OF INTEREST

Guarantor of the article: Louis Buscail, MD, PhD.
Specific author contributions: B. Bournet: study concept and design, including patients, collecting and interpreting the data, preparation and revision of the manuscript, has approved the final draft submitted; F. Muscari: including patients, collecting and interpreting the data, has approved the final draft submitted; C. Buscail: study concept and design, interpreting the data, revision of the manuscript, has approved the final draft submitted; E. Assenat: including patients, collecting the data, revision of the manuscript, has approved the final draft submitted; M. Barthet: including patients, collecting the data, has approved the final draft submitted; P. Hammel: including patients, collecting the data, revision of the manuscript, has approved the final draft submitted; J. Selves: performing molecular experiments, collecting and interpreting the data, revision of the manuscript, has approved the final draft submitted; R. Guimbaud: including patients, collecting the data, has approved the final draft submitted; P. Cordelier: performing molecular experiments, collecting and interpreting the data, revision of the manuscript, has approved the final draft submitted; L. Buscail: study concept and design, including patients, collecting and interpreting the data, preparation and revision of the manuscript, has approved the final draft submitted.
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Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Survival among patients with locally advanced and metastatic cancer patients remains poor.
- ✓ The activating point mutation of the *KRAS* oncogene on codon-12 is the major event in pancreatic adenocarcinoma.
- ✓ No molecular biomarker is available at present that can help or influence the management of pancreatic cancer patients.

WHAT IS NEW HERE

- ✓ The *KRAS* G12D mutation subtype is an independent prognostic marker for advanced pancreatic ductal carcinoma.
- ✓ This mutation also negatively influences the prognosis of patients treated by chemotherapy including gemcitabine-based regimen.

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