Pathol. Oncol. Res. (2019) 25:269–278
https://doi.org/10.1007/s12253-017-0341-0

ORIGINAL ARTICLE

Clinical Implications of NRAS Overexpression in Resectable Pancreatic Adenocarcinoma Patients

Javier Martinez-Useros[1] · Weiyao Li[1] · Tihomir Georgiev-Hristov[2] · Maria J. Fernandez-Aceñero[3] · Aurea Borrero-Palacios[1] · Nuria Perez[4] · Angel Celdran[2] · Jesus Garcia-Foncillas[1]

Received: 22 February 2017 / Accepted: 20 October 2017 / Published online: 3 November 2017

Abstract Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal forms of cancer, and its incidence is rising worldwide. Although survival can be improved by surgical resection, when detected at an early stage, this type of cancer is usually asymptomatic, and disease becomes only apparent after metastasis. Adjuvant treatment does not improve survival, thus after surgery there is a lack of predictive and prognosis biomarkers to predict treatment response and survival. The mitogen-activated protein-kinase and phosphoinositide 3-kinase signalling pathways play a crucial role in cancer development and progression. Especially, activated RAS proteins promote cell proliferation through constitutive stimulation of the downstream effectors RAF-MEK-ERK and PI3K-AKT. Mutational status of *NRAS* is required in several types of cancer like colorectal or cutaneous melanoma. However, mutations in this gene are very scarce in PDAC patients, and *NRAS* determination is not usually performed in clinical practice for this kind of tumor. In this study, we analyse the association between NRAS protein expression and progression-free survival and overall survival of an homogenous cohort of pancreatic ductal adenocarcinoma patients from a single-centre. Interestingly, we found that patients with high expression not only showed longer progression-free survival than those patients with low expression (22 *versus* 9 months, respectively) (*P* = 0.013), but also longer overall survival (43 *versus* 19 months, respectively) (*P* = 0.020). These results confirm NRAS expression could be used to differentiate patients according to their prognosis. Proportional hazard model revealed NRAS expression together with grade of differentiation as pathological variables to predict patient's outcome.

Keywords Pancreatic ductal adenocarcinoma · PDAC · NRAS · KRAS · TGCA · Biomarker · Progression-free survival · Overall survival · Grade of differentiation

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12253-017-0341-0) contains supplementary material, which is available to authorized users.

✉ Javier Martinez-Useros
javier.museros@oncohealth.eu

✉ Jesus Garcia-Foncillas
jesus.garciafoncillas@oncohealth.eu

[1] Translational Oncology Division, OncoHealth Institute, Fundacion Jimenez Diaz University Hospital, Autonomous University of Madrid, Av. Reyes Católicos 2, 28040 Madrid, Spain

[2] Hepatobiliary and Pancreatic Surgery Unit, General and Digestive Tract Surgery Department, Fundacion Jimenez Diaz University Hospital, Av. Reyes Católicos 2, 28040 Madrid, Spain

[3] Department of Pathology, Clinico San Carlos University Hospital, C/ Profesor Martin Lagos, 28040 Madrid, Spain

[4] Department of Pathology, University Hospital Fundacion Jimenez Diaz, Av. Reyes Católicos 2, 28040 Madrid, Spain

Introduction

Pancreatic ductal adenocarcinoma (PDAC) has higher incidence in the industrialised countries [1]. It is the fourth leading cause of cancer death in the USA in both sexes, and it is estimated that 48,960 new cases of PDAC were diagnosed in 2015 [2]. Moreover, it is the eighth leading cause of cancer deaths in men and the ninth among women worldwide [3].

It has been reported that the 5-year survival rate is 50% when tumors are <2 cm in size [4] and close to 100% for tumors <1 cm [5]. Thus, early detection of tumors is crucial to improve survival. However, pancreatic cancer is usually asymptomatic, and the disease only becomes apparent when tumors invade surrounding tissues or metastasises to distant

organs [6]. In fact, distant metastasis is found in 53% of pancreatic cancer patients at the time of diagnosis [2].

To date, surgical resection remains the best management option for pancreatic cancer originating in the ampulla of Vater, bile duct, or head of pancreas. Survival can be predicted based on pathological characteristics such as tumor size, grade of differentiation, and lymph-node status [7]. However, there is a lack of clinically validated prognostic or predictive markers that can be used in patient management after surgery [8], although several prognostic molecular biomarkers have been suggested, such as Smad4 or MUC1, predictive markers including SPARC, HuR, or members of the BRCA2 family [9, 10].

Up to now, preoperative levels of carbohydrate antigen 19–9 (CA 19–9) are the only prognostic biomarker approved by the Food and Drug Administration (FDA) for use in cases of resectable pancreatic cancer [11]. This marker shows a relatively high sensitivity and specificity for pancreatic cancer [12], providing superior results to those of other markers, such as carcinoembryonic antigen (CEA), carbohydrate antigen 50 (CA-50), and DUPAN-2 [13, 14]. However, the applicability of CA 19–9 is compromised by the fact that biliary obstruction can increase its serum levels [15], and up to 10% of the population cannot synthesise this antigen [16].

The mitogen-activated protein-kinase (MAPK) signalling pathway plays a crucial role in cancer development and progression. MAPK pathway is composed by EGFR-RAS-RAF-MEK-ERK factors [17]. Especially, activated RAS proteins promote cell proliferation through constitutive stimulation of the downstream RAF-MEK-ERK effectors [18]. RAS family are GTPases composed by Kirsten, Harvey and Neuroblastoma *RAS* genes (*KRAS, HRAS* and *NRAS,* respectively) that are subcategories of at least 35 related proteins [19, 20].

Genetic mutations in *RAS* genes can deregulate kinase activity and constitutively hyperactivate the MAPK pathway that eventually leads to tumorigenesis. In general, mutations that affect MAPK/ERK pathway are singular and independent events. Two different mutations are infrequently found in the RAS/RAF/MEK/ERK pathway within the same tumor [21].

NRAS mutation status is found in several types of cancers [17]. In melanoma the *NRAS* mutation is found from 15% to 29% of cases [22, 23]; 12% have been found in non-small cell lung cancer [24]; 41% in thyroid cancers [25]; and 10% in colorectal cancers [26] among other tumors. *KRAS* mutations serve as predictive biomarker of anti-EGFR monoclonal-based therapy response in colorectal cancer [27]. Concerning pancreatic cancer, *KRAS* mutations have been reported to occur very frequently ~90% of cases [28] and have been found in ~95% of earliest pre-neoplasic stages of pancreatic cancer [29]. Nevertheless, *NRAS* mutations are absent in pancreatic cancer patients [30]. It is now evident that alterations in copy-number of *KRAS* and *NRAS* may also occur. Amplification of *KRAS* has been found in 34% (10/29) of PDAC derived cell lines [31] and in ~5% of PDAC patients [32]. However, *NRAS* amplification or polisomy is rare (~1%) [32].

RAS proteins are needed to maintain cell stability and homeostasis of non-transformed cells. Normal MAPK function is also responsible for tumor suppression through induction of senescence and ubiquitinization and degradation of proteins that triggers cell cycle activity and survival [33, 34]. Moreover, RAS activation is able to degrade proteins required for both migration and tumor [33]. This fact points out other role of wild-type RAS proteins to avoid turn normal cells into tumor cells.

To date, outcome of resected PDAC patients is clinically predicted according to clinico-pathologic criteria and there is a lack of molecular biomarkers after surgical resection to predict prognosis.

Thus, the present study shows quite clearly a new role of NRAS protein as a prognosis biomarker for resectable pancreatic cancer patients and suggests the potential effect of NRAS as a protective factor for this deadful neoplasia.

Materials and Methods

Patient Samples

A total of 53 patients with PDAC who underwent pancreaticoduonenectomy from 2007 to 2013 at the Hepatobiliary and Pancreatic Surgery Unit (General and Digestive Tract Surgery Department, Fundación Jiménez Díaz University Hospital) were assessed for eligibility. Eight patients were excluded due to insufficient sample quality for immunohistochemistry, patients lost to follow-up, or tumors having origins other than the head of pancreas.

Statement of human rights Ethics approval and consent to participate: The Fundación Jiménez Díaz Institutional Review Board (IRB) evaluated the study, granting approval on December 9, 2014 with approval number 17/14. The clinical samples used in the study were kindly supplied by the Fundación Jiménez Díaz-Universidad Autónoma de Madrid BioBank (PT13/0010/0012). All patients gave written informed consent for the use of their biological samples for research purposes.

Tissue Microarray, Immunohistochemistry and Quantification

A tissue microarray (TMA) was conducted for immunohistochemistry analysis and contained 90 cores (2 cores per patient) using the MTA-1 tissue arrayer (Beecher Instruments, Sun Prairie, USA). Each core (diameter, 1 mm) was punched from pre-selected tumor regions in paraffin-embedded tissues. Staining was conducted in 2-μm sections. Slides were

deparaffinised by incubation at 60 °C for 10 min and incubated with PT-Link (Dako, Denmark) for 20 min at 95 °C in a high pH buffered solution. To block endogenous peroxidase, holders were incubated with peroxidase blocking reagent (Dako, Denmark). Biopsies were incubated for 20 min with 1:1000 dilution of NRAS antibody against a synthetic peptide of 15 amino acids from near the C terminus of Human NRAS isoform 1 (NP_002515) (ab167136; Abcam, Cambridge, UK) followed by incubation with the appropriate anti-Ig horseradish peroxidase-conjugated polymer (EnVision, Dako, Denmark) to detect antigen-antibody reaction. A human pancreatic tissue was used as a positive control for immunohistochemical staining (according to the human protein atlas available at http://www.proteinatlas.org). Sections were then visualised with 3,3′-diaminobenzidine as a chromogen for 5 min and counterstained with haematoxylin. Photographs were taken with a stereo microscope (Leica DMi1, Wetzlar, Germany). Immunoreactivity was quantified blind as tertiles according to the intensity of positively stained cells (Fig. 1). Quantification for each patient biopsy was calculated with the average of both cores by two independent pathologists.

Statistical Analysis

The association between NRAS expression and progression-free survival after resection was the primary endpoint, and overall survival was the secondary endpoint. Progression-free survival was defined as the interval between the dates of surgery and recurrence (local or distant). Overall survival was defined as the interval between the dates of surgery and death from any cause. Survival curves were generated using the Kaplan-Meier method, and significant differences in survival between groups were determined by the log-rank test.

The association between UNR expression and clinico-pathological variables was evaluated by Chi-square or Fisher exact tests.

The Cox proportional hazards model was used to assess the hazard ratios and confidence intervals of both molecular and clinical variables. Thus, only statistically significant variables found in the univariate analysis were included in the multivariate analysis. *P*-values ≤0.05 were considered significant. Analysis was performed with the IBM SPSS programme, version 20.0.

TCGA-Pancreatic Cancer Dataset Analysis

A dataset of 186 pancreatic adenocarcinoma patients from TCGA (The Cancer Genome Atlas) and obtained from cBioportal [35, 36] were eligible for the study. We selected patients with complete sequence and copy-number analysis (n = 149/186). NRAS protein expression Z-scores were available on 100/149 patients of this dataset and were obtained by

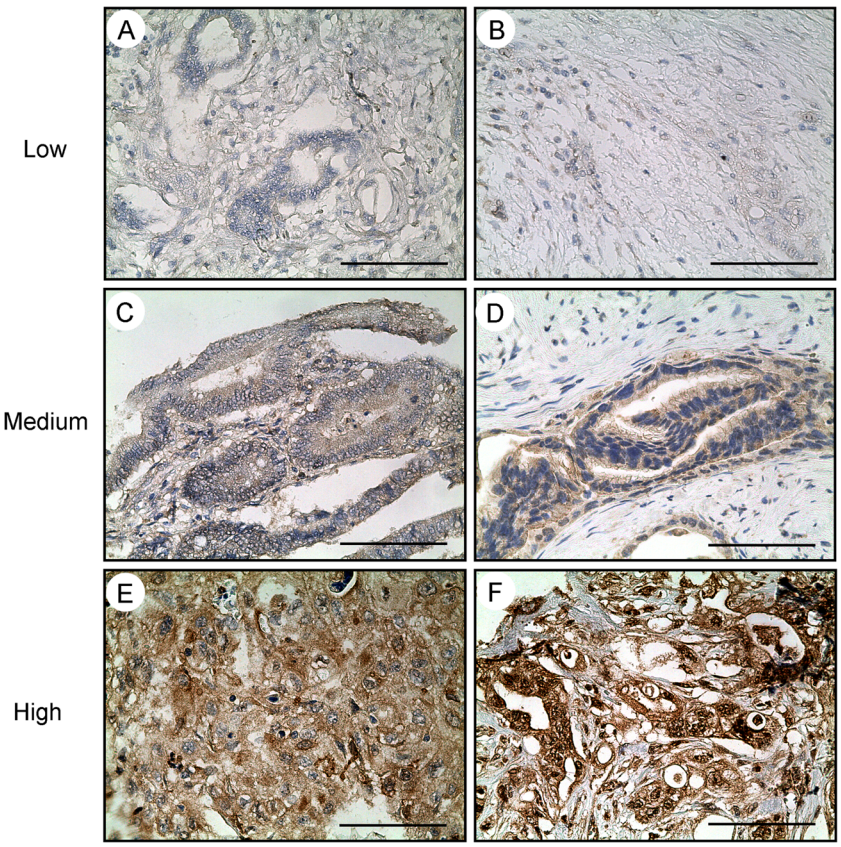

Fig. 1 Representative micrographs of PDAC patients samples with low NRAS expression (**a** and **b**), medium expression (**c** and **d**), and high expression (**e** and **f**). Scale bar: 110 μM

reverse-phase protein array. NRAS mRNA expression Z-scores were available in 149/149 patients and were obtained by RNASeq Version 2 [37]. The Z-score threshold for both protein and mRNA NRAS expression was +/− 1.96 (for *P*-value <0.05, and 95% Confidence Interval with two tailed distribution).

Mutation data from PDAC patients was extracted from whole exome sequencing.

Putative copy-number variation was determined using GISTIC 2.0. Values were considered as follows: −2 = homozygous deletion; −1 = hemizygous deletion; 0 = neutral / no change; 1 = gain; 2 = high level amplification.

The association between NRAS protein or mRNA expression and mutation status of *KRAS* or *NRAS*, and copy-number variation of *KRAS* or *NRAS* was analysed with Fisher exact test. Statistical analysis was performed with the IBM SPSS programme, version 20.0. *P*-values ≤0.05 were considered significant.

Results

Patient Characteristics

Our cohort was composed by 60% of female patients. The median age of patients was 66 years (range, 37–82 years). Pathologic diagnosis revealed the size of the resected tumors to be lower than 3 cm in 82% of cases. Twenty percent of tumors were stage I and 80% stage II, of them 62% were stage IIB. Patients were confirmed to be low-grade resectable PDAC in 76% of cases according to the recommendations of the College of American Pathologists [38]. Negative surgical margins were found after surgery in 85% of cases. And 65 % of patients showed lymph-node involvement and most patients had neural and vascular invasion (71% and 69%, respectively). Adjuvant treatment based on gemcitabine alone or gemcitabine plus radiotherapy was administered post-surgery in 40% of patients based on consensus of a multidisciplinary team. Gemcitabine was administered in 3–12 cycles depending on radiotherapy doses (45–54 Gy in 1.8–2.5 Gy fractions). Nevertheless, adjuvant treatment did not impact neither in progression-free survival ($P = 0.921$, data not shown), nor in overall survival ($P = 0.899$, data not shown). The clinical features of the PDAC patients included in the study are summarised in Table 1.

Table 1 Clinico-pathological characteristics of resectable PDAC patients enrolled in the study

Characteristics	N (%)
Age	
<65 years	20 (44%)
>65 years	25 (56%)
Sex	
Female	27 (60%)
Male	18 (40%)
Size	
<3 cm	37 (82%)
>3 cm	5 (11%)
N/A	3 (7%)
Stage	
IA	6 (13%)
IB	3 (7%)
IIA	8 (18%)
IIB	28 (62%)
Grade	
Low grade	34 (76%)
High grade	11 (24%)
Lymph nodes involved	
No	15 (33%)
Yes	29 (65%)
N/A	1 (2%)
Adjuvant treatment	
No	25 (56%)
Yes	18 (40%)
N/A	2 (4%)
Positive margins	
No	38 (85%)
Yes	7 (15%)
Origin	
Pancreas	23 (51%)
Biliar Duct	12 (27%)
Ampulla	10 (22%)
Vascular invasion	
No	14 (31%)
Yes	31 (69%)
Neural invasion	
No	13 (29%)
Yes	32 (71%)

N/A not available

High NRAS Expression is Associated to Better Clinical Outcome in PDAC Patients

Due to importance of NRAS in development of several neoplasias we decided to study the role of this factor in this kind of patients. *NRAS* mutations are rare in PDAC patients [30]; thus, we determined NRAS in PDAC patients by immunohistochemistry.

All positive stained samples exhibited a cytoplasmic expression pattern (Fig. 1) and some diffuse membrane localisation, especially in some cases with medium or high expression levels (Fig. 1d, e and f). Subsequently, patient's samples were stratified into three groups as low, medium or high, according to NRAS intensity of expression (Fig. 1).

The association between NRAS expression and outcome was assessed. Survival analysis in terms of progression-free survival showed statistically significant differences between three arms ($P = 0.025$) (Fig. 2a). Interestingly, it was observed that patients stratified according to low and medium expression levels had similar behaviour according to progression-free survival, while patients from high expression presented a clearly better outcome. Therefore, high expression arm was established as cut-off point for NRAS expression. This new stratification achieved higher statistical power and indicated high NRAS expression as better outcome event in PDAC patients ($P = 0.013$) (Fig. 2b). Median analysis revealed that patients with low-medium NRAS expression took them 9 months to experience disease progression (range, 7–11 months). However, those patients with high expression took over 22 months to develop disease progression (range, 8–35 months).

Overall survival according to NRAS expression was analysed as a secondary endpoint. Kaplan-Meier curves with low, medium and high NRAS expression did not achieve statistical significance ($P = 0.052$) (Fig. 3a). Then, we grouped low and medium NRAS expression arms to compare with high NRAS expression. Here, statistical significant differences were found between both cohorts and high NRAS expression that showed longer overall survival and a subsequent better outcome ($P = 0.020$) (Fig. 3b). Median overall survival of patients with low and medium NRAS expression was 15 months (range, 8–22 months) while median was not reached for high NRAS expression arm. While mean overall survival for high expression was found to be twice longer than mean of low and medium NRAS expression cohort. Albeit, mean overall survival was found to be 19 months (range, 12–26 months) for low and medium expression while high NRAS expression patients took an average of 43 months until death (range, 34–52 months). The results obtained validates NRAS expression thus to be used to differentiate PDAC patients according to their better or poor prognosis.

In order to compare the potential prognosis value of NRAS expression with the other clinico-pathological variables we performed a Cox proportional hazards model analysis (Table 2). Then, univariate analysis revealed NRAS expression as statistical significant factor for both progression-free survival (HR = 0.326; 95%CI = 0.128–0.837; *P = 0.020*) and overall survival (HR = 0.268; 95%CI = 0.082–0.881; *P = 0.030*) together with tumor stage. High grade appeared to be a poor prognosis factor for both progression-free survival (HR = 3.256; 95%CI = 1.283–8.266; *P = 0.013*) and overall survival (HR = 3.832; 95%CI = 1.374–10.688; *P = 0.010*). In the multivariate analysis for progression-free survival both variables stayed statistically significant. Thus, NRAS expression (HR = 0.328; 95%CI = 0.127–0.852; *P = 0.022*) could be used to predict progression-free survival in PDAC patients together with tumor grade (HR = 3.492; 95%CI = 1.327–9.188; *P = 0.011*). Unfortunately, only tumor grade remained statistically significant in multivariate analysis for overall survival (Table 2).

To verify if expression of NRAS expression could be associated to any clinico-pathological variable registered in the study, a crosstab was performed thereafter (Table 3). However, no statistically significant association was found in this analysis that included age ($P = 0.807$), gender ($P = 0.555$), adjuvant treatment ($P = 0.678$), tumor origin

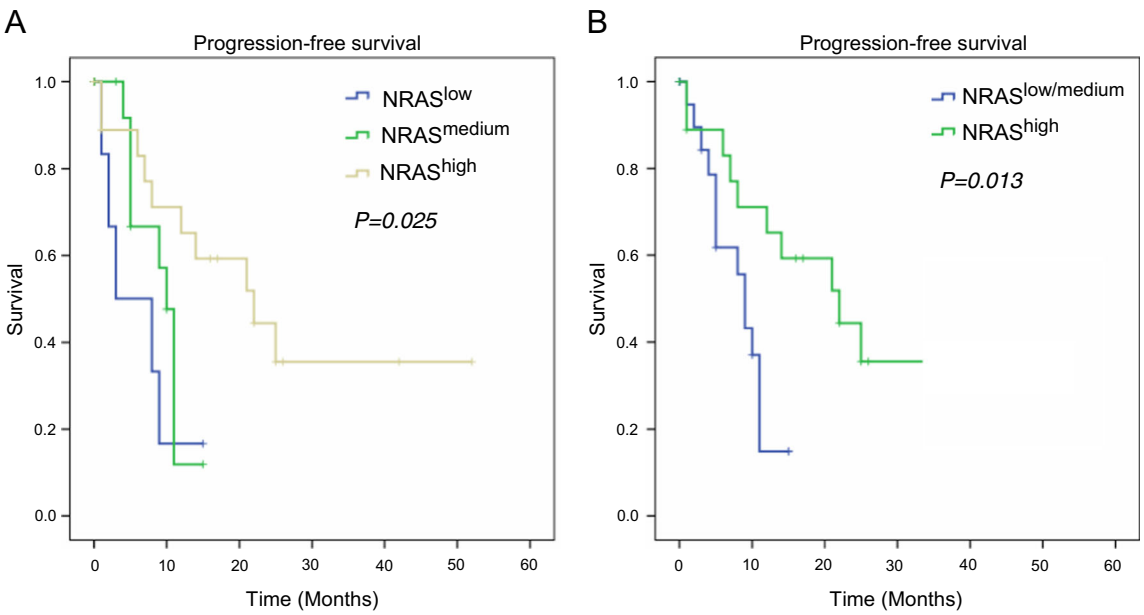

Fig. 2 NRAS expression predicts longer progression-free survival. **a** Kaplan-Meier analysis for progression-free survival after surgery of patients stratified into three groups as low (blue line), medium (green line) or high (yellow line), according to NRAS intensity of expression. **b** Kaplan-Meier analysis for progression-free survival with high NRAS expression (green line) established as cut-off point to separate patients into high- and low risk. *P*-values ≤0.05 were considered significant

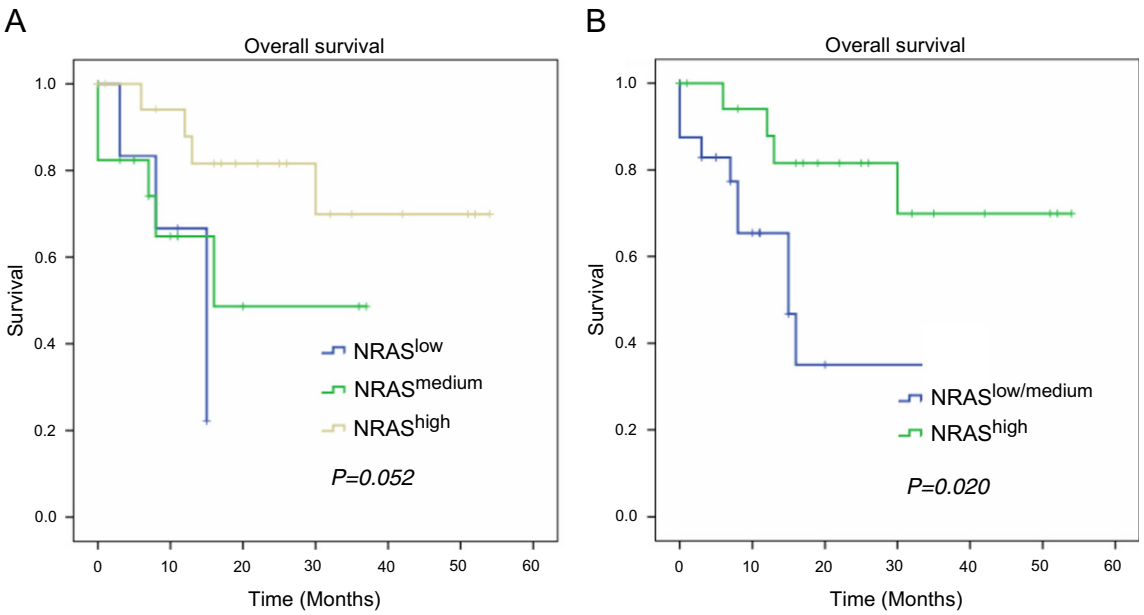

Fig. 3 NRAS expression predicts longer overall survival. **a** Kaplan-Meier analysis for overall survival of patients stratified into three groups as low (blue line), medium (green line) or high (yellow line), according to NRAS intensity of expression. **b** Kaplan-Meier analysis for overall survival with high NRAS expression (green line) established as cut-off point to separate patients into risk groups. *P*-values ≤0.05 were considered significant

Table 2 Cox uni- and multivariate analysis with molecular and clinical variables on progression-free survival and overall survival PDAC patients

	PFS Univariate				OS Univariate			
	HR	95% Lower	CI Upper	*P*	HR	95% Lower	CI Upper	*P*
Age				0.832				0.239
>65 years *vs* <65 years	1.091	0.488	2.441		1.892	0.654	5.469	
Sex				0.896				0.592
Male *vs* Female	1.057	0.461	2.400		1.374	0.430	4.392	
Adjuvant treatment				0.925				0.898
No	1.000				1.000			
Gemcitabine	0.952	0.351	2.581		0.926	0.268	3.194	
Gemcitabine + RT	1.154	0.385	3.455		0.716	0.159	3.219	
Origin				0.953				0.648
Pancreas	1.000				1.000			
Biliar Duct	1.071	0.395	2.908		1.137	0.360	3.587	
Ampulla	1.186	0.398	3.537		0.551	0.113	2.684	
Size				0.737				0.456
>3 cm *vs* <3 cm	1.233	0.363	4.183		1.647	0.444	6.114	
Grade				0.013				0.010
High *vs* Low	3.256	1.283	8.266		3.832	1.374	10.688	
Stage				0.159				0.187
II *vs* I	2.389	0.712	8.022		3.926	0.514	29.971	
Positive margins				0.856				0.183
Yes *vs* No	1.120	0.331	3.783		2.202	0.689	7.035	
Vascular Invasion				0.876				0.912
Yes *vs* No	1.077	0.425	2.725		1.068	0.334	3.410	
Neural Invasion				0.906				0.878
Yes *vs* No	1.058	0.418	2.678		1.095	0.343	3.498	
Lymph nodes affected				0.445				0.166
Yes *vs* No	1.406	0.575	3.439		2.885	0.645	12.909	
NRAS				0.020				0.030
High *vs* Low	0.326	0.128	0.837		0.268	0.082	0.881	
Grade				0.011				0.029
High *vs* Low	3.492	1.327	9.188		3.389	1.137	10.103	
NRAS				0.022				0.091
High *vs* Low	0.328	0.127	0.852		0.345	0.101	1.184	

PFS progression-free survival, *OS* overall survival, *HR* hazard ratio, *CI* confidence interval, *RT* radiotherapy, *vs* versus.

(P = 0.738), grade (P = 0.190), stage (P = 0.673), positive margins of resection (P = 0.363), lymph nodes involvement (P = 0.186), vascular invasion (P = 0.373) or neural invasion (P = 0.190). Intriguingly, NRAS expression showed a trend towards significance with tumor size (P = 0.093) (Table 3).

Since genomic aberrations like mutations or variation in *NRAS* copy number may occur in PDAC, although to a lesser extent, we wonder if could be a link between NRAS expression and genomic alterations. Taking into consideration the scarce number of patients of our cohort and the low rate of *NRAS* alterations in PDAC, we decided to evaluate the association between NRAS expression and alterations of *NRAS* using a public PDAC database from TGCA in order to found more positive cases with *NRAS* alterations. This dataset comprises 149 patients with complete genomic information from a total of 186 patients. Unfortunately, these analyses could not be assessed because neither of the patients presented *NRAS* mutation (n = 0/149) nor alteration in copy number (n = 0/149).

Due *KRAS* alterations are more frequent in PDAC, we wonder if NRAS expression could be linked to mutation or amplification of *KRAS*. The incidence of *KRAS* mutation was 91% (n = 135/149), and G12D was the most numerous with 41% of positive cases (n = 61/149), followed by G12 V (26%, n = 39/149), G12R (18%, n = 27/149), Q61H (4%, n = 6/149), Q61R (1%, n = 2/149), and others like G12A, G12C, G12S or G13C that were present in very low incidence (<1%, n = 1/149 each). The association between *KRAS* mutation and NRAS expression (protein or mRNA) revealed no statistically significant association (P = 0.593 and P = 0.694, respectively) (Supplementary Table 1).

Copy-number variation analysis of *KRAS* showed amplification in 4% of patients (n = 6/149). Fisher exact test performed with *KRAS* amplification and NRAS protein or mRNA expression did not achieve statistical significance (P = 1.000 and P = 0.586, respectively) (Supplementary Table 2).

Table 3 Association between NRAS expression and clinico-pathological parameters

Clinico-pathological parameters	P
Age	0.807
Gender	0.555
Adjuvant treatment	0.678
Origin	0.738
Size	0.093
Grade	0.190
Stage	0.673
Positive margins	0.363
Lymph nodes involvement	0.186
Vascular invasion	0.373
Neural invasion	0.190

Discussion

PDAC is one of the most deadful cancers worldwide because tumor cells tend to metastasize vital organs what reduces survival significantly. Surgical resection is currently considered the best option so far to improve survival. Not every patient is a good candidate for this procedure, though. [39]. The survival rate of resected patients reaches 3.5 years, however it decreases to 0.8 years in non-operated patients. Therefore, overall mean life expectancy is 1.4 years (P < 0.001) [40]. Adjuvant therapy is usually based on 5FU or gemcitabine and combined with radiation therapy according to clinical guidelines depending on the clinical and pathologic characteristics [41, 42]. But, as we have checked in our cohort of patients, adjuvant therapy has a scarce benefit in survival, and it is mostly used as a palliative intent [43, 44]. Therefore, unresectable patients with limited treatment options are encouraged to participate in clinical trials at any stage of disease.

The current FDA-approved marker for PDAC, CA19–9, is not recommended for its use in disease recurrence nor for response to therapy prediction [45]. Several pre- and clinical studies have suggested the potential use of *BRCA2* mutations as biomarkers for platimun-based chemotherapy and PARP inhibitors [10]. However, secondary genomic alterations will prompt patients to acquired resistance [46]. It is therefore imperative to find new treatments, predictive tools and prognostic biomarkers to improve survival.

IHC is an easy, cost-effective and reliable technique commonly used in clinical practice to determine not only protein expression but also *RAS* mutations with high sensitivity and specificity of antibodies [47]. Thus, we decided to determine NRAS expression by this method.

Our group has recently reported NRAS expression in 31 low-grade PDAC patients and a high trend towards significance was found between NRAS expression and both progression-free survival (P = 0.054) and overall survival (P = 0.092) [48]. Then, we increased the number of PDAC patients, and we included not only low-grade but also high-grade patients for the present study. Interestingly, survival analyses according NRAS expression achieved statistical significance for both progression-free and overall survival. Cox proportional hazards model confirmed the potential role of NRAS as a prognosis biomarker for progression-free survival together with tumor grade. Unfortunately, NRAS expression had not enough statistical power when compared to tumor grade for overall survival prediction. These results not only support NRAS expression as a prognosis biomarker of progression-free survival but also set NRAS at the same level that tumor grade to predict patients' prognosis above other clinico-pathological variables like stage or lymph node involvement.

To date, NRAS prognosis value is based on its oncogenic mutational status. *KRAS* and *NRAS* mutations activate RAF-

MEK-ERK effectors through constitutive stimulation of MAPK pathway and the subsequent cell proliferation [17].

Both *KRAS* and *NRAS* mutation status has become an essential tool in the clinical guidelines for the use of anti-EGFR treatments for colorectal cancer patients [49]. It is estimated that ~35% of colorectal cancer patients carry *KRAS* mutation, however *NRAS* mutation is only found in ~4% [50]. The incidence of *NRAS* mutations is higher in cutaneous melanoma (~15%) and plays an important role in melanocyte homeostasis [51]. Since genomic alterations of *NRAS* could appear in PDAC, although in very low incidence [30, 32, 52], we decided to study the link between NRAS expression and *NRAS* mutation or copy-number variation. One of the limitations of our study was the low number of patients recruited, and then it could be hardy to found positive cases for *NRAS* mutation and/or genomic amplification. Therefore, we decided to study the link between NRAS expression and *NRAS* genomic alterations in a public repository (TGCA) with higher number of patients and with availability of expression profile and genomic information. Unfortunately, genomic alterations in *NRAS* were absent in this dataset. In contrast, *KRAS* mutation and amplification was found in 91% and in 4% of patients respectively. However, no statistical association was found between NRAS expression and *KRAS* mutation or amplification.

To the best of our knowledge, this is the first time NRAS expression plays a protective effect against cancer. In fact, several scientific reports support the opposite but none dealing with PDAC. Nras down-regulation by miR-340 and miR-143 has been reported to be a good prognosis event in glioblastoma [53, 54]. Moreover, increased expression of NRAS has been associated to Vemurafenib resistance in melanoma derived cell lines [55, 56]. Low expression of wild-type NRAS showed better response rates to dacarbazine and longer progression-free survival in metastatic melanoma patients [57].

It has been reported that NRAS present 5 different isoforms with differential activation or repression potential of the downstream pathways in both normal and tumor tissues such as lung, thyroid, skin, and colon [58]. Isoforms 1 and 5 present an increased ability to phosphorilate both downstream pathways RAF-MEK-ERK and PI3K-AKT. Isoform 2 activates phosphorilation of PI3K but not RAF. In contrast, isoforms 3 and 4 present a reduced phosphorilation capability of the both pathways RAF-MEK-ERK and PI3K-AKT. Of them all, isoform 5 is the more aggressive variant [58]. In our study, we have determined expression of NRAS isoform 1 in PDAC patients. Thus, according to the results it is suggested that the increased expression of isoform 1 has a protective effect in such disease. We cannot compare between results because PDAC has not been included in the above-mentioned report. So, further research is necessary to verify activation of MAPK and PI3K pathways in PDAC.

Overall, the results presented here suggest a clear association between NRAS expression and a better prognosis. Then, NRAS expression could be a potential biomarker of better outcome in PDAC.

Acknowledgements We thank Oliver Shaw, PhD (FIIS-FJD) for editing the manuscript for English usage, clarity, and style. We also A. Cazorla MD, PhD for a double-blind tissue immunostainings evaluation and quantification.

Author's Contribution J.M.-U. designed the study, analyse data, draft the article and is the guarantor for the article; W.L. and A.B.P. performed experiments; T.G.-H. and M.J.F.-A. performed patients database; N.P., A.C. and J.G.-F. revised critically the manuscript.FundingThis work has been carried out with Spanish Health Research Project Funds PI16/01468 from "Instituto de Salud Carlos III FEDER" (J.G.-F.), of the Spanish Ministry of Economy, Industry and Competitiveness.

Compliance with Ethical Standards

Conflicts of Interest None to declare.

References

1. Hariharan D, Saied A, Kocher HM (2008) Analysis of mortality rates for pancreatic cancer across the world. HPB 10(1):58–62
2. Siegel RL, Miller KD, Jemal A (2016) Cancer statistics, 2016. CA Cancer J Clin 66(1):7–30. https://doi.org/10.3322/caac.21332
3. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA Cancer J Clin 61(2):69–90
4. Egawa S, Takeda K, Fukuyama S, Motoi F, Sunamura M, Matsuno S (2004) Clinicopathological aspects of small pancreatic cancer. Pancreas 28(3):235–240
5. Ariyama J, Suyama M, Satoh K, Sai J (1998) Imaging of small pancreatic ductal adenocarcinoma. Pancreas 16(3):396–401
6. Kelsen DP, Portenoy R, Thaler H, Tao Y, Brennan M (1997) Pain as a predictor of outcome in patients with operable pancreatic carcinoma. Surgery 122(1):53–59
7. Hidalgo M (2010) Pancreatic cancer. N Engl J Med 362(17):1605–1617. https://doi.org/10.1056/NEJMra0901557
8. Stathis A, Moore MJ (2010) Advanced pancreatic carcinoma: current treatment and future challenges. Nat Rev Clin Oncol 7(3):163–172. https://doi.org/10.1038/nrclinonc.2009.236
9. Fong ZV, Winter JM (2012) Biomarkers in pancreatic cancer: diagnostic, prognostic, and predictive. Cancer J 18(6):530–538. https://doi.org/10.1097/PPO.0b013e31827654ea
10. Martinez-Useros J, Garcia-Foncillas J (2016) The Role of BRCA2 Mutation Status as Diagnostic, Predictive, and Prognosis Biomarker for Pancreatic Cancer. Biomed Res Int 2016:1869304. https://doi.org/10.1155/2016/1869304
11. Maithel SK, Maloney S, Winston C, Gonen M, D'Angelica MI, Dematteo RP, Jarnagin WR, Brennan MF, Allen PJ (2008) Preoperative CA 19-9 and the yield of staging laparoscopy in patients with radiographically resectable pancreatic adenocarcinoma. Ann Surg Oncol 15(12):3512–3520. https://doi.org/10.1245/s10434-008-0134-5
12. Goonetilleke KS, Siriwardena AK (2007) Systematic review of carbohydrate antigen (CA 19-9) as a biochemical marker in the diagnosis of pancreatic cancer. Eur J Surg Oncol 33(3):266–270. https://doi.org/10.1016/j.ejso.2006.10.004
13. Bosman FTCF, Hruban RH, Theise ND (eds) (2010) World Health Organization Classification of Tumours of the Digestive System. IARC Press, Lyon

14. Klöppel GHR, Longnecker DS, Adler G, Kern SE, Partanen TJ (2000) Ductal adenocarcinoma of the pancreas. World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Digestive System. IARC Press, Lyon
15. Kim JE, Lee KT, Lee JK, Paik SW, Rhee JC, Choi KW (2004) Clinical usefulness of carbohydrate antigen 19-9 as a screening test for pancreatic cancer in an asymptomatic population. J Gastroenterol Hepatol 19(2):182–186
16. Kawai S, Suzuki K, Nishio K, Ishida Y, Okada R, Goto Y, Naito M, Wakai K, Ito Y, Hamajima N (2008) Smoking and serum CA19-9 levels according to Lewis and secretor genotypes. Int J Cancer 123(12):2880–2884. https://doi.org/10.1002/ijc.23907
17. Burotto M, Chiou VL, Lee JM, Kohn EC (2014) The MAPK pathway across different malignancies: a new perspective. Cancer 120(22):3446–3456. https://doi.org/10.1002/cncr.28864
18. Kolch W (2005) Coordinating ERK/MAPK signalling through scaffolds and inhibitors. Nat Rev Mol Cell Biol 6(11):827–837. https://doi.org/10.1038/nrm1743
19. Colicelli J (2004) Human RAS superfamily proteins and related GTPases. Sci STKE 2004(250):RE13. https://doi.org/10.1126/stke.2502004re13
20. Malumbres M, Barbacid M (2003) RAS oncogenes: the first 30 years. Nat Rev Cancer 3(6):459–465. https://doi.org/10.1038/nrc1097
21. Roberts PJ, Der CJ (2007) Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene 26(22):3291–3310. https://doi.org/10.1038/sj.onc.1210422
22. Colombino M, Capone M, Lissia A, Cossu A, Rubino C, De Giorgi V, Massi D, Fonsatti E, Staibano S, Nappi O, Pagani E, Casula M, Manca A, Sini M, Franco R, Botti G, Caraco C, Mozzillo N, Ascierto PA, Palmieri G (2012) BRAF/NRAS mutation frequencies among primary tumors and metastases in patients with melanoma. J Clin Oncol 30(20):2522–2529. https://doi.org/10.1200/JCO.2011.41.2452
23. Edlundh-Rose E, Egyhazi S, Omholt K, Mansson-Brahme E, Platz A, Hansson J, Lundeberg J (2006) NRAS and BRAF mutations in melanoma tumours in relation to clinical characteristics: a study based on mutation screening by pyrosequencing. Melanoma Res 16(6):471–478. https://doi.org/10.1097/01.cmr.0000232300.22032.86
24. Seo JS, Ju YS, Lee WC, Shin JY, Lee JK, Bleazard T, Lee J, Jung YJ, Kim JO, Yu SB, Kim J, Lee ER, Kang CH, Park IK, Rhee H, Lee SH, Kim JI, Kang JH, Kim YT (2012) The transcriptional landscape and mutational profile of lung adenocarcinoma. Genome Res 22(11):2109–2119. https://doi.org/10.1101/gr.145144.112
25. Bansal M, Gandhi M, Ferris RL, Nikiforova MN, Yip L, Carty SE, Nikiforov YE (2013) Molecular and histopathologic characteristics of multifocal papillary thyroid carcinoma. Am J Surg Pathol 37(10):1586–1591. https://doi.org/10.1097/PAS.0b013e318292b780
26. Cancer_Genome_Atlas_Network (2012) Comprehensive molecular characterization of human colon and rectal cancer. Nature 487(7407):330–337. https://doi.org/10.1038/nature11252
27. Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Cote JF, Tomasic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F, Laurent-Puig P (2006) KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res 66(8):3992–3995. https://doi.org/10.1158/0008-5472.CAN-06-0191
28. Smit VT, Boot AJ, Smits AM, Fleuren GJ, Cornelisse CJ, Bos JL (1988) KRAS codon 12 mutations occur very frequently in pancreatic adenocarcinomas. Nucleic Acids Res 16(16):7773–7782
29. Kanda M, Matthaei H, Wu J, Hong SM, Yu J, Borges M, Hruban RH, Maitra A, Kinzler K, Vogelstein B, Goggins M (2012) Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia. Gastroenterology 142(4):730–733 e739. https://doi.org/10.1053/j.gastro.2011.12.042
30. Mitsuhashi K, Nosho K, Sukawa Y, Matsunaga Y, Ito M, Kurihara H, Kanno S, Igarashi H, Naito T, Adachi Y, Tachibana M, Tanuma T, Maguchi H, Shinohara T, Hasegawa T, Imamura M, Kimura Y, Hirata K, Maruyama R, Suzuki H, Imai K, Yamamoto H, Shinomura Y (2015) Association of Fusobacterium species in pancreatic cancer tissues with molecular features and prognosis. Oncotarget 6(9):7209–7220. 10.18632/oncotarget.3109
31. Hamidi H, Lu M, Chau K, Anderson L, Fejzo M, Ginther C, Linnartz R, Zubel A, Slamon DJ, Finn RS (2014) KRAS mutational subtype and copy number predict in vitro response of human pancreatic cancer cell lines to MEK inhibition. Br J Cancer 111(9):1788–1801. https://doi.org/10.1038/bjc.2014.475
32. Biankin AV, Waddell N, Kassahn KS, Gingras MC, Muthuswamy LB, Johns AL, Miller DK, Wilson PJ, Patch AM, Wu J, Chang DK, Cowley MJ, Gardiner BB, Song S, Harliwong I, Idrisoglu S, Nourse C, Nourbakhsh E, Manning S, Wani S, Gongora M, Pajic M, Scarlett CJ, Gill AJ, Pinho AV, Rooman I, Anderson M, Holmes O, Leonard C, Taylor D, Wood S, Xu Q, Nones K, Fink JL, Christ A, Bruxner T, Cloonan N, Kolle G, Newell F, Pinese M, Mead RS, Humphris JL, Kaplan W, Jones MD, Colvin EK, Nagrial AM, Humphrey ES, Chou A, Chin VT, Chantrill LA, Mawson A, Samra JS, Kench JG, Lovell JA, Daly RJ, Merrett ND, Toon C, Epari K, Nguyen NQ, Barbour A, Zeps N, Kakkar N, Zhao F, Wu YQ, Wang M, Muzny DM, Fisher WE, Brunicardi FC, Hodges SE, Reid JG, Drummond J, Chang K, Han Y, Lewis LR, Dinh H, Buhay CJ, Beck T, Timms L, Sam M, Begley K, Brown A, Pai D, Panchal A, Buchner N, De Borja R, Denroche RE, Yung CK, Serra S, Onetto N, Mukhopadhyay D, Tsao MS, Shaw PA, Petersen GM, Gallinger S, Hruban RH, Maitra A, Iacobuzio-Donahue CA, Schulick RD, Wolfgang CL, Morgan RA, Lawlor RT, Capelli P, Corbo V, Scardoni M, Tortora G, Tempero MA, Mann KM, Jenkins NA, Perez-Mancera PA, Adams DJ, Largaespada DA, Wessels LF, Rust AG, Stein LD, Tuveson DA, Copeland NG, Musgrove EA, Scarpa A, Eshleman JR, Hudson TJ, Sutherland RL, Wheeler DA, Pearson JV, McPherson JD, Gibbs RA, Grimmond SM (2012) Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. Nature 491(7424):399–405. https://doi.org/10.1038/nature11547
33. Deschenes-Simard X, Gaumont-Leclerc MF, Bourdeau V, Lessard F, Moiseeva O, Forest V, Igelmann S, Mallette FA, Saba-El-Leil MK, Meloche S, Saad F, Mes-Masson AM, Ferbeyre G (2013) Tumor suppressor activity of the ERK/MAPK pathway by promoting selective protein degradation. Genes Dev 27(8):900–915. https://doi.org/10.1101/gad.203984.112
34. Serrano M, Lin AW, ME MC, Beach D, Lowe SW (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88(5):593–602
35. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C, Schultz N (2013) Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 6(269):pl1. https://doi.org/10.1126/scisignal.2004088
36. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C, Schultz N (2012) The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2(5):401–404. https://doi.org/10.1158/2159-8290.CD-12-0095
37. Li B, Ruotti V, Stewart RM, Thomson JA, Dewey CN (2010) RNA-Seq gene expression estimation with read mapping uncertainty. Bioinformatics 26(4):493–500. https://doi.org/10.1093/bioinformatics/btp692
38. Adsay NV, Basturk O, Bonnett M, Kilinc N, Andea AA, Feng J, Che M, Aulicino MR, Levi E, Cheng JD (2005) A proposal for a

new and more practical grading scheme for pancreatic ductal adenocarcinoma. Am J Surg Pathol 29(6):724–733
39. Wagner M, Redaelli C, Lietz M, Seiler CA, Friess H, Buchler MW (2004) Curative resection is the single most important factor determining outcome in patients with pancreatic adenocarcinoma. Br J Surg 91(5):586–594. https://doi.org/10.1002/bjs.4484
40. Cucchetti A, Ercolani G, Taffurelli G, Serenari M, Maroni L, Pezzilli R, Del Gaudio M, Ravaioli M, Cescon M, Pinna AD (2016) A comprehensive analysis on expected years of life lost due to pancreatic cancer. Pancreatology 16(3):449–453
41. Ducreux M, Cuhna AS, Caramella C, Hollebecque A, Burtin P, Goere D, Seufferlein T, Haustermans K, Van Laethem JL, Conroy T, Arnold D (2015) Cancer of the pancreas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 56: v56–v68
42. Tempero MA MM, Al-Hawary M, Behrman SW, Benson A1, Berlin JD, Cha C, Chiorean EG, Chung V, Cohen SJ, Czito B, Dillhoff M, Feng M, Ferrone CR, Hardacre J, Hawkins W, Herman J, Hoffman JP, Ko AH, Komanduri S, Koong A, Lowy AM, Ma WW, Moravek C, Mulvihill SJ, Nakakura EK, O'Reilly EM, Obando J, Reddy S, Thayer S, Weekes CD, Wolff RA, Wolpin BM, Burns J, Darlow S (2016) Pancreatic Adenocarcinoma Version 1.2016. NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®) National Comprehensive Cancer Network
43. Abbruzzese JL (2002) New applications of gemcitabine and future directions in the management of pancreatic cancer. Cancer 95(4 Suppl):941–945. https://doi.org/10.1002/cncr.10753
44. Li D, Xie K, Wolff R, Abbruzzese JL (2004) Pancreatic cancer. Lancet 363(9414):1049–1057. https://doi.org/10.1016/S0140-6736(04)15841-8
45. Winter JM, Yeo CJ, Brody JR (2013) Diagnostic, prognostic, and predictive biomarkers in pancreatic cancer. J Surg Oncol 107(1): 15–22
46. Sakai W, Swisher EM, Karlan BY, Agarwal MK, Higgins J, Friedman C, Villegas E, Jacquemont C, Farrugia DJ, Couch FJ, Urban N, Taniguchi T (2008) Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. Nature 451(7182):1116–1120
47. Kakavand H, Walker E, Lum T, Wilmott JS, Selinger CI, Smith E, Saw RP, Yu B, Cooper WA, Long GV, O'Toole SA, Scolyer RA (2016) BRAF(V600E) and NRAS(Q61L/Q61R) mutation analysis in metastatic melanoma using immunohistochemistry: a study of 754 cases highlighting potential pitfalls and guidelines for interpretation and reporting. Histopathology 69(4):680–686. https://doi.org/10.1111/his.12992
48. Martinez-Useros J, Georgiev-Hristov T, Fernandez-Acenero MJ, Borrero-Palacios A, Indacochea A, Guerrero S, Li W, Cebrian A, Gomez Del Pulgar T, Puime-Otin A, Del Puerto-Nevado L, Rodriguez-Remirez M, Perez N, Celdran A, Gebauer F, Garcia-Foncillas J (2017) UNR/CDSE1 expression as prognosis biomarker in resectable pancreatic ductal adenocarcinoma patients: A proof-of-concept. PLoS One 12(8):e0182044. https://doi.org/10.1371/journal.pone.0182044
49. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, Kalogeras KT, Kotoula V, Papamichael D, Laurent-Puig P, Penault-Llorca F, Rougier P, Vincenzi B, Santini D, Tonini G, Cappuzzo F, Frattini M, Molinari F, Saletti P, De Dosso S, Martini M, Bardelli A, Siena S, Sartore-Bianchi A, Tabernero J, Macarulla T, Di Fiore F, Gangloff AO, Ciardiello F, Pfeiffer P, Qvortrup C, Hansen TP, Van Cutsem E, Piessevaux H, Lambrechts D, Delorenzi M, Tejpar S (2010) Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol 11(8):753–762. https://doi.org/10.1016/S1470-2045(10)70130-3
50. Palomba G, Doneddu V, Cossu A, Paliogiannis P, Manca A, Casula M, Colombino M, Lanzillo A, Defraia E, Pazzola A, Sanna G, Putzu C, Ortu S, Scartozzi M, Ionta MT, Baldino G, Sarobba G, Capelli F, Sedda T, Virdis L, Barca M, Gramignano G, Budroni M, Tanda F, Palmieri G (2016) Prognostic impact of KRAS, NRAS, BRAF, and PIK3CA mutations in primary colorectal carcinomas: a population-based study. J Transl Med 14(1):292. https://doi.org/10.1186/s12967-016-1053-z
51. Hodis E, Watson IR, Kryukov GV, Arold ST, Imielinski M, Theurillat JP, Nickerson E, Auclair D, Li L, Place C, Dicara D, Ramos AH, Lawrence MS, Cibulskis K, Sivachenko A, Voet D, Saksena G, Stransky N, Onofrio RC, Winckler W, Ardlie K, Wagle N, Wargo J, Chong K, Morton DL, Stemke-Hale K, Chen G, Noble M, Meyerson M, Ladbury JE, Davies MA, Gershenwald JE, Wagner SN, Hoon DS, Schadendorf D, Lander ES, Gabriel SB, Getz G, Garraway LA, Chin L (2012) A landscape of driver mutations in melanoma. Cell 150(2):251–263. https://doi.org/10.1016/j.cell.2012.06.024
52. Prior IA, Lewis PD, Mattos C (2012) A comprehensive survey of Ras mutations in cancer. Cancer Res 72(10):2457–2467. https://doi.org/10.1158/0008-5472.CAN-11-2612
53. Fiore D, Donnarumma E, Roscigno G, Iaboni M, Russo V, Affinito A, Adamo A, De Martino F, Quintavalle C, Romano G, Greco A, Soini Y, Brunetti A, Croce CM, Condorelli G (2016) miR-340 predicts glioblastoma survival and modulates key cancer hallmarks through down-regulation of NRAS. Oncotarget 7(15):19531–19547. 10.18632/oncotarget.6968
54. Wang L, Shi ZM, Jiang CF, Liu X, Chen QD, Qian X, Li DM, Ge X, Wang XF, Liu LZ, You YP, Liu N, Jiang BH (2014) MiR-143 acts as a tumor suppressor by targeting N-RAS and enhances temozolomide-induced apoptosis in glioma. Oncotarget 5(14): 5416–5427. 10.18632/oncotarget.2116
55. Lidsky M, Antoun G, Speicher P, Adams B, Turley R, Augustine C, Tyler D, Ali-Osman F (2014) Mitogen-activated protein kinase (MAPK) hyperactivation and enhanced NRAS expression drive acquired vemurafenib resistance in V600E BRAF melanoma cells. J Biol Chem 289(40):27714–27726. https://doi.org/10.1074/jbc.M113.532432
56. Nazarian R, Shi H, Wang Q, Kong X, Koya RC, Lee H, Chen Z, Lee MK, Attar N, Sazegar H, Chodon T, Nelson SF, McArthur G, Sosman JA, Ribas A, Lo RS (2010) Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. Nature 468(7326):973–977. https://doi.org/10.1038/nature09626
57. Birkeland E, Busch C, Berge EO, Geisler J, Jonsson G, Lillehaug JR, Knappskog S, Lonning PE (2013) Low BRAF and NRAS expression levels are associated with clinical benefit from DTIC therapy and prognosis in metastatic melanoma. Clin Exp Metastasis 30(7):867–876. https://doi.org/10.1007/s10585-013-9587-4
58. Eisfeld AK, Schwind S, Hoag KW, Walker CJ, Liyanarachchi S, Patel R, Huang X, Markowitz J, Duan W, Otterson GA, Carson WE 3rd, Marcucci G, Bloomfield CD, de la Chapelle A (2014) NRAS isoforms differentially affect downstream pathways, cell growth, and cell transformation. Proc Natl Acad Sci U S A 111(11):4179–4184. https://doi.org/10.1073/pnas.1401727111