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Dysregulation of KRAS signaling in pancreatic cancer is not associated with *KRAS* mutations and outcome

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Abstract. Pancreatic ductal adenocarcinoma (PDAC) is a tumor with a poor prognosis, and no targeted therapy is currently available. The aim of the present study was to investigate the prognostic significance of the expression of V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*), downstream signaling pathway genes and the association with clinical characteristics in PDAC patients undergoing radical surgery. Tumors and adjacent non-neoplastic pancreatic tissues were examined in 45 patients with histologically verified PDAC. *KRAS* and B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) gene mutation analysis was performed using the *KRAS*/*BRAF*/phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α array. The transcript profile of 52 KRAS downstream signaling pathway genes was assessed using quantitative-polymerase chain reaction. *KRAS* mutation was detected in 80% of cases. The genes of four signaling pathways downstream of KRAS, including the phosphoinositide 3-kinase/3-phosphoinositide-dependent protein kinase 1/V-akt murine thymoma viral oncogene homolog 1, RAL guanine nucleotide exchange factor, Ras and Rab interactor 1/*ABL* proto-oncogene-1, non-receptor tyrosine kinase, and RAF proto-oncogene serine/threonine-protein kinase/mitogen-activated protein kinase pathways, exhibited differential expression in PDAC compared with that in the adjacent normal tissues. However, no significant differences in expression were evident between patients with *KRAS*-mutated and wild-type tumors. The expression of KRAS downstream signaling pathways genes did not correlate with angioinvasion, perineural invasion, grade or presence of lymph node metastasis. Additionally, the presence of *KRAS* mutations was not associated with overall survival. Among the KRAS downstream effective signaling pathways molecules investigated, only v-raf-1 murine leukemia viral oncogene homolog 1 expression was predictive of prognosis. Overall, *KRAS* mutation is present in the majority of cases of PDAC, but is not associated with changes in the expression of KRAS downstream signaling pathways and the clinical outcome. This may partly explain the failure of KRAS-targeted therapies in PDAC.

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Key words: pancreatic ductal adenocarcinoma, KRAS, gene expression, mutation, overall survival

Introduction

Pancreatic ductal adenocarcinoma (PDAC; Online Mendelian Inheritance in Man no. 260350) ranks fourth in the leading causes of cancer-associated mortality in Western countries (1). Despite diagnostic and therapeutic advances, the prognosis of PDAC remains poor. Only 20% of patients present with potentially resectable disease at the time of diagnosis, while due to the high propensity for tumor recurrence, the 5-year overall survival (OS) rate in patients undergoing surgery with radical intent is usually <25%. Although a number of prospective clinical trials have demonstrated that adjuvant systemic therapy improves the patient outcome following surgery, adjuvant chemotherapy appears to be effective only in a minority of patients, and the majority of the patients ultimately succumb to the disease. The prognosis of metastatic patients is extremely poor, with a median OS time of <1 year (2). Consequently, novel regimens of adjuvant treatment are being investigated and there is currently no definitive standard of adjuvant therapy.

In PDAC, mutations in the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) gene occur in 75-90% of cases, representing the most frequent, as well as the earliest, genetic alteration. *KRAS* mutations, specifically in codons 12 and 13, lead to constitutive activation of downstream signaling pathways that are important for tumor initiation, development and spread (3,4). KRAS signaling is highly complex and dynamic, with various downstream effector pathways interconnected at different levels by cross-signaling and feedback loops (5). The four major KRAS downstream pathways reported in PDAC are RAF proto-oncogene serine/threonine-protein kinase (RAF)/mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase 1 (PDK1)/*ABL* proto-oncogene-1, non-receptor tyrosine kinase (ABL), RAL guanine nucleotide exchange factor, and Ras and Rab interactor 1 (RIN1)/ABL pathways (Fig. 1) (6-10). This multiplicity of downstream pathways may partly explain the failure of existing efforts to target epidermal growth factor receptor, KRAS or serine/threonine-protein kinase B-raf (BRAF) using specific inhibitors, underlining the complexity of genetic changes and the resistance of the cancer cells.

The aim of the present study was to assess the association between gene expression from the four major KRAS-effective pathways in PDAC and the clinical features of the patients, and to evaluate the potential predictive and prognostic significance.

Materials and methods

Patients. A cohort of 45 consecutive patients with PDAC who underwent surgery with curative intent was recruited from two oncology centers in the Czech Republic (Institute of Clinical and Experimental Medicine, Prague; and University Hospital, Masaryk University, Brno, Czech Republic) between August 2008 and January 2012. Inclusion criteria were: i) Adult operable patients with suspected pancreatic carcinoma based on clinical imaging methods; ii) patients who provided informed consent; and iii) pancreatic carcinoma diagnosis was verified by collaborating pathologist. None of the patients had received prior chemotherapy. Characteristics of the patient cohort are summarized in Table I. The tissue specimen collection and processing, and the data retrieval were as described previously (11).

All patients signed an informed consent form, in accordance with the requirements for ethical approval, which was provided by the Institutional Review Boards of the Institute of Clinical and Experimental Medicine and University Hospital, Masaryk University, Brno.

Isolation of nucleic acids and cDNA synthesis. Tissue samples were homogenized and total RNA and DNA was isolated as previously described (12,13). cDNA was synthesized using 0.5 μg total RNA and characterized as previously described (14). cDNA was then pre-amplified by TaqMan® PreAmp Master mix to enrich the specific targets for gene expression analysis using TaqMan Gene Expression assays (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) (Table II). The cDNA pre-amplification was performed with 5 μl cDNA using 14 pre-amplification cycles (10 min at 95˚C and 14 cycles of 15 sec at 95˚C), and the pre-amplification uniformity of cDNA was checked according to the procedure recommended by the manufacturer (Thermo Fisher Scientific, Inc.).

Quantitative polymerase chain reaction (qPCR). qPCR was performed using the ViiA7 Real-Time PCR system using TaqMan Gene Expression assays (Table I), with optimized primer and probe sets and TaqMan Gene Expression Master mix (Thermo Fisher Scientific, Inc.). Processing of precursor 4, *S. cerevisiae*, homolog of, mitochondrial ribosomal protein L19, E74-like factor 1 and eukaryotic translation initiation factor 2B subunit 1 were used as reference genes for studies of gene expression in human pancreatic carcinoma based on our previously published data (15). Determination of transcript levels was performed exactly as previously described (10) and the qPCR study adhered to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines (16).

KRAS and BRAF mutation status. *KRAS* and *BRAF* gene mutation analysis was performed using the *KRAS*/*BRAF*/phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (*PIK3CA*) (KBP) Array (EV3799A/B; Randox Laboratories Ltd., Crumlin, Northern Ireland) according to the manufacturer's instructions. The assay is based on a combination of multiplex PCR and biochip array hybridization for high discrimination between multiple wild-type and mutant DNA regions in the *KRAS* (mutations in codons 12, 13 and 61), *BRAF* (V600E mutation) and *PIK3CA* (mutations in codons 542, 545 and 1,047) genes. Providing there are enough copies of DNA present, ~1% of mutants can readily be detected in a background of wild-type genomic DNA. A unique primer set is designed for each mutation target (and control), which will hybridize to a complementary discrete test region (DTR) on the biochip array. Each DTR corresponds to a particular mutation target. One of the target-specific primers in each pair contains a biotin label, which on addition of streptavidin-horseradish peroxidase conjugate permits chemiluminescence detection of hybridized products on the biochip array. Dedicated software processes produced automatic results.

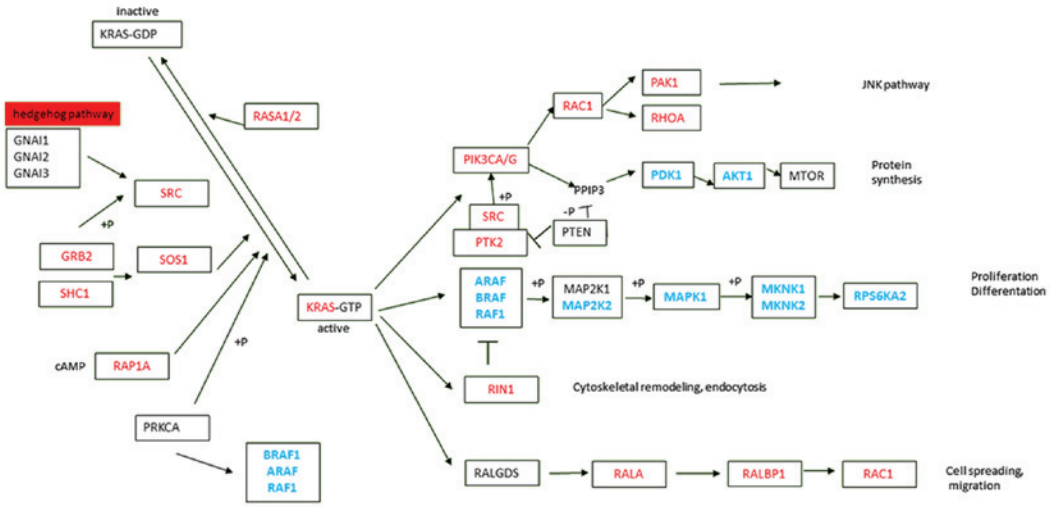


Figure 1. Dysregulation of KRAS signaling pathway in PDAC tumors compared with that in the paired adjacent non-malignant tissues. KRAS pathway map noting the differentially expressed genes in PDAC tumors compared with those in adjacent non-malignant tissues. Genes overexpressed in tumor tissue are in red, while downregulated genes are in blue. Genes not exhibiting differential expression are in black. KRAS, KRAS proto-oncogene, GTPase; PDAC, pancreatic ductal adenocarcinoma.

Statistical analysis. Differences in gene expression profiles between tumor and paired non-neoplastic control tissues and between wild-type and KRAS-mutated samples were evaluated using the RT^2 Profiler PCR Assay Data Analysis v3.5 program (Qiagen GmbH, Hilden, Germany). This gene expression analysis suite performs fold-change calculations from raw quantification cycle values for reference and target genes based on the ΔΔCq method described by Livak and Schmittgen (17), and enables hierarchical clustering of gene expression profiles between the compared groups of patients and data. Differences in intratumoral gene expression levels between patients stratified by clinical data were evaluated by the Kruskal-Wallis test.

OS was defined as the time between the date of surgery and all-cause mortality. Surviving patients were censored at the last follow-up in December 2015. Patients were divided into two groups by the median intratumoral gene expression levels of individual genes and the survival functions were computed by the Kaplan-Meier method, with statistical significance evaluated by the Breslow test using SPSS v16.0 (SPSS, Inc., Chicago, IL, USA).

P<0.05 was considered to indicate a statistically significant difference. All P-values are departures from two-sided tests. The correction for multiple testing was applied according to the Bonferroni and the false discovery rate (FDR) methods.

Results

Study population. The study was performed on 45 patients with resected (R0 resection in >90% of cases) PDAC who had not received any prior neoadjuvant therapy. Overall, 80% (36/45) of patients harbored *KRAS* mutations in the DNA of the tumor tissue, while *BRAF* mutations were not found in any sample (Table II). The majority of patients (76%; n=34) received adjuvant chemotherapy consisting of nucleoside analogs (gemcitabine and/or 5-fluorouracil).

The median OS time was 23.7 months, with 18% of patients (n=8) alive at the time of data cut off (December 2015).

Table I. Characteristics of the patient cohort.

Variables	20
Mean age (range), years	63.9 (46-80)
Sex, n (%)	
Male	20 (44.4)
Female	25 (55.6)
Histological grade, n (%)	
G1+G2 (well to moderate)	30 (66.7)
G3+G4 (poor)	15 (33.3)
Primary tumor extent of invasion, n (%)	
pT1 tumor limited to the pancreas ≤2 cm	1 (2.2)
pT2 tumor limited to the pancreas >2 cm	5 (11.1)
pT3 tumor extending beyond the pancreas	39 (86.7)
Regional lymph nodes, n (%)	
pN0	17 (37.8)
pN1	28 (62.2)
KRAS mutations in codons 12 and 13[a], n (%)	
Wild-type (GGTGGC)	9 (20.0)
G12V (GTTGGC)	10 (22.2)
G12D (GATGGC)	15 (33.3)
G12R (CGTGGC)	6 (13.3)
Other (G13D, Q61R, Q61H)	3 (6.7)
Not assessed	2 (4.4)
BRAF mutations[a], n (%)	
Wild-type (GTG)	43 (95.6)
V600E (GAG)	0 (0.0)
Not assessed	2 (4.4)
Patient status at the data cut off, n (%)	
Deceased	37 (82.2)
Alive	8 (17.8)

[a]Base changes are underlined. BRAF, B-Raf proto-oncogene, serine/threonine kinase; KRAS, KRAS proto-oncogene, GTPase.

Table II. List of TaqMan gene expression assays used in the study.

Gene abbreviation	Gene name	Assay ID
AKT1	V-akt murine thymoma viral oncogene homolog 1	Hs00178289_m1
AKT2	V-akt murine thymoma viral oncogene homolog 2	Hs01086102_m1
ARAF	V-raf murine sarcoma viral oncogene homolog 1	Hs00176427_m1
BRAF	V-raf murine sarcoma viral oncogene homolog B1	Hs00269944_m1
GRB2	Growth factor receptor-bound protein 2	Hs00257910_s1
GSK3B	Glycogen synthase kinase 3-β	Hs00275656_m1
KRAS	V-ki-ras2 Kirsten rat sarcoma viral oncogene homolog	Hs00364284_g1
MAP2K1	Mitogen-activated protein kinase kinase 1	Hs00983247_g1
MAP2K2	Mitogen-activated protein kinase kinase 2	Hs04194606_gH
MAP2K7	Mitogen-activated protein kinase kinase 7	Hs00178198_m1
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	Hs00394890_m1
MAP3K2	Mitogen-activated protein kinase kinase kinase 2	Hs01109981_m1
MAP3K4	Mitogen-activated protein kinase kinase kinase 4	Hs00245958_m1
MAP3K7	Mitogen-activated protein kinase kinase kinase 7	Hs01105682_m1
MAPK1	Mitogen-activated protein kinase 1	Hs01046830_m1
MAPK10	Mitogen-activated protein kinase 10	Hs00373455_m1
MAPK14	Mitogen-activated protein kinase 14	Hs01051152_m1
MAPK3	Mitogen-activated protein kinase 3	Hs00946872_m1
MAPK7	Mitogen-activated protein kinase 7	Hs00611114_g1
MAPK8	Mitogen-activated protein kinase 8	Hs00177083_m1
MAPK9	Mitogen-activated protein kinase 9	Hs00177102_m1
MKNK1	Mitogen-activated protein kinase-interacting serine/threonine kinase 1	Hs00374376_m1
MKNK2	Mitogen-activated protein kinase-interacting serine/threonine kinase 2	Hs01046586_g1
MTOR	Mechanistic target of rapamycin	Hs00234508_m1
PAK1	p21 protein-activated kinase 1	Hs00176815_m1
PDPK1	3-phosphoinositide-dependent protein kinase 1	Hs00176884_m1
PIK3CA	Phosphatidylinositol 3-kinase, catalytic, α	Hs00907966_m1
PIK3CG	Phosphatidylinositol 3-kinase, catalytic, γ	Hs00277090_m1
PLK3	Polo-like kinase 3	Hs00177725_m1
PRKACA	Protein kinase, camp-dependent, catalytic, α	Hs00427274_m1
PRKCA	Protein kinase c, α	Hs00925195_m1
PTEN	Phosphatase and tensin homolog	Hs02621230_s1
PTK2	Protein-tyrosine kinase, cytoplasmic	Hs01056457_m1
PTK2B	Protein-tyrosine kinase 2, β	Hs01559708_m1
RAC1	Ras-related C3 botulinum toxin substrate 1	Hs01025984_m1
RAF1	V-raf-1 murine leukemia viral oncogene homolog 1	Hs00234119_m1
RALA	V-ral simian leukemia viral oncogene homolog A	Hs01564991_g1
RALBP1	RalA-binding protein 1	Hs01034988_g1
RALGDS	Ral guanine nucleotide dissociation stimulator	Hs00325141_m1
RAP1A	Ras-related protein 1A	Hs01092205_g1
RASA1	Ras p21 protein activator 1	Hs00963555_m1
RASA2	Ras p21 protein activator 2	Hs01003325_m1
RHOA	Ras homolog gene family, member A	Hs00357608_m1
RIN1	Ras and rab interactor 1	Hs00182870_m1
RPS6KA2	Ribosomal protein S6 kinase, 90-kd, 2	Hs00179731_m1
RPS6KA4	Ribosomal protein S6 kinase, 90-kd, 4	Hs00177670_m1
RPS6KA5	Ribosomal protein S6 kinase, 90-kd, 5	Hs01046594_m1
SHC1	SHC transforming protein	Hs01050699_g1
SOS1	Son of sevenless, *Drosophila*, homolog 1	Hs00362316_m1
SOS2	Son of sevenless, *Drosophila*, homolog 2	Hs00412876_g1
SRC	V-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene	Hs01082238_g1
STAT3	Signal transducer and activator of transcription 3	Hs01047580_m1

Table II. Continued.

Gene abbreviation	Gene name	Assay ID
ELF1[a]	E74-like factor 1	Hs00152844_m1
EIF2B1[a]	Eukaryotic translation initiation factor 2B, subunit 1	Hs00426752_m1
MRPL19[a]	Mitochondrial ribosomal protein l19	Hs00608519_m1
POP4[a]	Processing of precursor 4, *S. cerevisiae*, homolog of	Hs00198357_ml

[a]Reference genes.

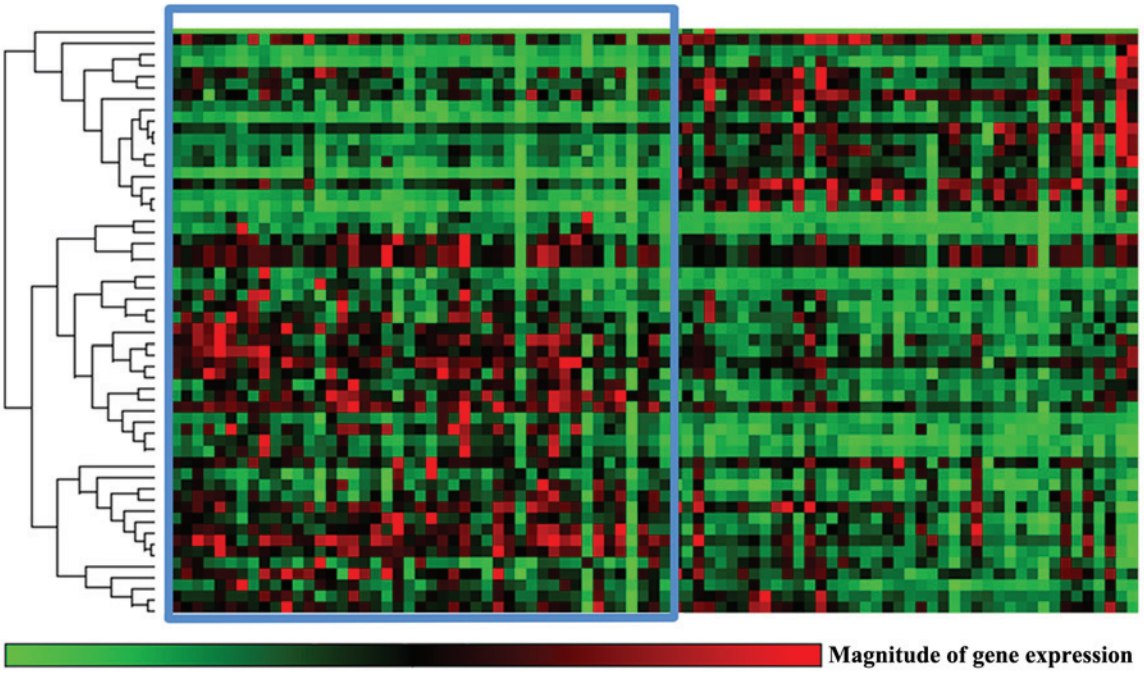


Figure 2. Heat map of KRAS proto-oncogene, GTPase signaling pathway expression profile of pancreatic ductal adenocarcinoma. Tumor samples are contained within the blue box on the left and the non-malignant pancreatic tissue samples are shown on the right.

Transcript levels of KRAS signaling pathways genes in PDAC. Considering the pivotal role of KRAS oncogene in the integration and transduction of mitogenic and metabolic signals, the transcript levels of 52 genes covering four major pathways downstream of *KRAS* were measured (Table I). The KRAS pathway was significantly dysregulated in tumors compared with that in adjacent non-malignant pancreatic tissues (Fig. 2; Table III). Significant overexpression of genes of the *PI3K/PDK1/AKT*, *RAL* guanine nucleotide exchange factor, and *RIN1/ABL* [phosphatidylinositol 3-kinase, catalytic, α/γ (*PIK3CA/G)*, p21 protein-activated kinase 1, V-ral simian leukemia viral oncogene homolog A, RalA-binding protein 1, Ras-related C3 botulinum toxin substrate 1, *RIN1*, protein-tyrosine kinase, cytoplasmic, and V-src avian sarcoma *(Schmidt-Ruppin A-2)* viral oncogene] pathways were observed, leading to cytoskeletal remodeling, endocytosis, cell spreading and migration (Table III; Fig. 1). By contrast, genes of the *RAF/MAPK* pathway exhibited significantly lower expression in tumors compared with that in the paired adjacent non-malignant pancreatic tissues (particularly in genes *ARAF*, *BRAF*, *V-RAF-1* murine leukemia viral oncogene homolog 1 (*RAF1*), mitogen-activated protein kinase kinase, mitogen-activated protein kinase 1, mitogen-activated protein kinase-interacting serine/threonine kinase 1/2 (*MKNK1/2)* and ribosomal protein S6 kinase, 90-kd, 2. All these results remained significant after FDR adjustment for multiple testing and the majority remained significant after Bonferroni correction (Table III; Fig. 1).

However, no association between *KRAS* downstream signaling pathway gene expression and tumor characteristics, including tumor size, grade, angioinvasion, lymph node metastasis or perineural invasion, passed the significance threshold of the Bonferroni test.

Impact of KRAS mutation status on transcript levels of target genes. From the 80% of tumor samples with *KRAS* mutations, the most common mutation, $KRAS^{G12D}$, was present in 33% (n=15) of the tumors. Only 1 tumor was found with a mutation in codon 13, and 2 cases with a mutation in codon 61 (Table II).

Patients divided by the *KRAS* mutation status significantly differed in terms of the gene expression of 5 of

Table III. Dysregulation of KRAS pathway genes in pancreatic ductal adenocarcinoma tumors in comparison to paired adjacent non-malignant tissues.

Gene	Fold-change[a] (tumor vs. non-malignant tissue)	95% confidence interval[a]	P-value[a]
AKT1	0.73	(0.63-0.83)	<0.001[b]
ARAF	0.72	(0.63-0.81)	<0.001[b]
BRAF	0.84	(0.74-0.93)	0.001
GRB2[c]	1.37	(1.04-1.69)	<0.001[b]
KRAS[c]	2.04	(1.67-2.41)	<0.001[b]
MAP2K2	0.64	(0.46-0.82)	<0.001[b]
MAP2K7	0.52	(0.39-0.65)	<0.001[b]
MAP3K1	0.85	(0.75-0.95)	0.010
MAP3K2[c]	1.24	(1.13-1.36)	<0.001[b]
MAP3K7[c]	1.28	(1.14-1.41)	<0.001[b]
MAPK1	0.77	(0.59-0.94)	<0.001[b]
MAPK14[c]	1.27	(1.14-1.40)	<0.001[b]
MAPK3[c]	1.71	(1.26-2.15)	<0.001[b]
MAPK7[c]	1.20	(1.01-1.38)	0.006
MAPK8	0.81	(0.74-0.88)	<0.001[b]
MAPK9	0.47	(0.38-0.55)	<0.001[b]
MKNK1	0.31	(0.25-0.38)	<0.001[b]
MKNK2	0.35	(0.26-0.44)	<0.001[b]
PAK1[c]	1.27	(1.08-1.45)	0.001
PDPK1	0.73	(0.64-0.81)	<0.001[b]
PIK3CA[c]	1.46	(1.25-1.68)	<0.001[b]
PIK3CG[c]	2.22	(1.61-2.82)	<0.001[b]
PLK3[c]	1.56	(1.23-1.88)	<0.001[b]
PTEN[c]	1.29	(1.05-1.53)	0.006
PTK2B[c]	1.68	(1.44-1.91)	<0.001[b]
RAC1[c]	1.65	(1.34-1.96)	<0.001[b]
RAF1	0.62	(0.54-0.69)	<0.001[b]
RALA[c]	1.43	(1.27-1.59)	<0.001[b]
RALBP1[c]	1.60	(1.39-1.81)	<0.001[b]
RAP1A[c]	1.18	(1.07-1.29)	<0.001[b]
RASA1[c]	1.28	(1.12-1.43)	<0.001[b]
RASA2[c]	1.87	(1.51-2.23)	<0.001[b]
RHOA[c]	1.23	(1.13-1.34)	<0.001[b]
RIN1[c]	1.39	(1.10-1.67)	0.002
RPS6KA2	0.65	(0.49-0.81)	0.001[b]
RPS6KA4[c]	1.76	(1.45-2.08)	<0.001[b]
SHC1[c]	1.24	(1.09-1.38)	0.001[b]
SOS1[c]	1.32	(1.14-1.50)	<0.001[b]
SOS2	0.68	(0.59-0.77)	<0.001[b]
SRC[c]	1.43	(1.16-1.71)	<0.001[b]

[a]Fold-change, 95% confidence interval and P-values were calculated using RT^2 Profiler PCR Assay Data analysis v3.5 program; [b]result that passed Bonferroni's correction for 52 analyzed genes (cut off P=0.001); [c]upregulated genes. There were 14 additional genes whose expression was not statistically significantly changed and that are therefore not listed in the table: *AKT2, GSK3B, MAP2K1, MAP3K4, MAPK10, MTOR, PRKACA, PRKCA, PTEN, PTK2, RALGDS, RAP1A, RPS6KA* and *STAT3*.

the 52 analyzed genes [*BRAF*, mitogen-activated protein kinase kinase kinase 4, mitogen-activated protein kinase 8, *MKNK1* and son of sevenless, *Drosophila*, homolog 2 (*SOS2*; P<0.05; Table IV)], but none of these associations passed the threshold for the multiple testing correction. The expression profiles of the KRAS signaling pathway as a whole also did not significantly differ between *KRAS* wild-type and *KRAS*-mutated tumors (Fig. 3).

Table IV. Downregulation of KRAS pathway genes in PDAC *KRAS*-mutated tumors compared with cases with wild-type *KRAS*.

Gene	Fold-change[a] (tumor vs. non-tumor)	95% confidence interval[a]	P-value[a]
BRAF	0.84	(0.72-0.95)	0.021
MAP3K4	0.79	(0.67-0.91)	0.035
MAPK8	0.84	(0.71-0.97)	0.027
MKNK1	0.72	(0.45-0.99)	0.033
SOS2	0.77	(0.59-0.94)	0.003

[a]Fold-change, 95% confidence interval and P-values were calculated using RT^2 Profiler PCR Assay Data analysis v3.5 program.

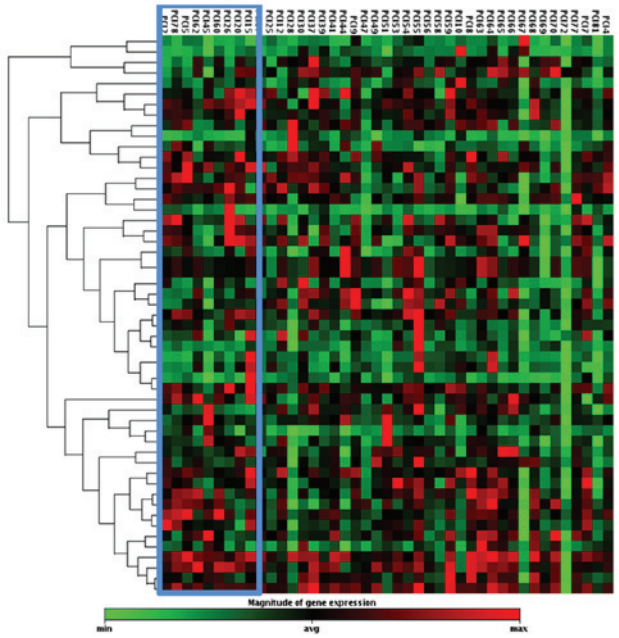


Figure 3. Heat map of KRAS signaling pathway expression profile of *KRAS*-mutated pancreatic ductal adenocarcinoma. *KRAS* wild-type tumors are marked by the blue box on the left and the samples harboring *KRAS* mutations are on the right. KRAS, KRAS proto-oncogene, GTPase.

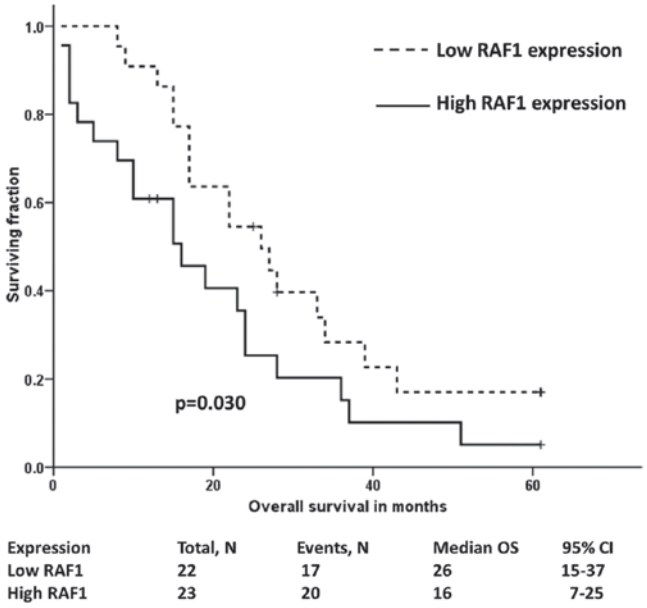


Figure 4. Kaplan-Meier survival plot presenting the correlation between *RAF1* expression and OS. RAF1, V-RAF-1 murine leukemia viral oncogene homolog 1; OS, overall survival; CI, confidence interval.

KRAS mutation status had no significant effect on the OS time of the PDAC patients. *KRAS* wild-type patients experienced a median OS time of 22.3 months, and patients with *KRAS* mutation experienced a median OS time of 21.0 months (P=0.182).

There was also no association between *KRAS* mRNA transcript levels and OS time. In contrast to the rest of pathway, *RAF1* showed a significant association with the OS time of the PDAC patients. Patients with *RAF1* expression levels lower than the median experienced longer OS times than patients with higher *RAF1* expression levels (P=0.030) (Fig. 4). However, this association did not pass Bonferroni correction for multiple testing.

Discussion

Mutation analysis of the present cohort of patients with operable PDAC aligns with that of prior studies reporting the presence of *KRAS* mutation in the majority of PDAC cases (18,19). Additionally, the genes of four KRAS downstream signaling pathways, including the *PI3K/PDK1/AKT*, *RAL* guanine nucleotide exchange factor, *RIN1/ABL* and *RAF/MAPK* pathways, exhibited differential expression in PDAC compared with that of the adjacent normal tissues, although no significant differences were observed in the expression of these genes between patients with *KRAS*-mutated and wild-type tumors. The expression profiles of KRAS downstream signaling pathways were not associated with pathological characteristics that reflect tumor biology, including angioinvasion, perineural invasion, grade or presence of lymph node metastasis.

Similar to earlier studies (20-22), the present data indicated that in this cohort of patients (with early-stage disease and following radical surgery) the presence of a *KRAS* mutation had no effect on the OS time of the patients, although there was limited power to determine associations indicating more minor effects due to the limited size of the patient cohort. Moreover, with the exception of RAF1, no impact was observed of the expression profile of the KRAS downstream major effective signaling pathways on OS. These findings may explain why all previous efforts targeting KRAS failed to improve the patient outcome.

Despite sustained efforts in preclinical and clinical research, PDAC remains a malignancy with an almost uniformly fatal prognosis (23). In contrast to other solid tumors, there has been no major progress in the systemic therapy of PDAC during the last decade. In particular, there is currently no targeted agent with clinically significant activity against this tumor.

Although molecular biomarkers play a crucial role in the management of numerous solid tumors (24), there are currently no useful biomarkers for treatment selection in PDAC. In recent years, a number of negative trials of targeted therapy have been conducted in PDAC (25,26). Consequently, there is an urgent requirement to improve the understanding of PDAC pathogenesis and biology in order to identify novel therapeutic approaches and to define subgroups of patients for tailored therapies. It has been demonstrated that *KRAS* mutations represent the driver mutations in the majority of PDAC cases. KRAS-targeted agents can be classified into several categories according to the mechanism of action, namely small-molecule RAS-binding ligands, inhibitors of KRAS membrane anchorage, inhibitors that bind to RAS-binding domains of RAS-effector proteins and inhibitors of KRAS expression (27). However, attempts to therapeutically target KRAS or the downstream pathways have all thus far failed in clinical trials (28-32).

In conclusion, as expected, *KRAS* was mutated in the majority of PDAC cases. The genes of the KRAS downstream signaling pathways, including the *PI3K/PDK1/AKT*, *RAL* guanine nucleotide exchange factor, *RIN1/ABL* and *RAF/MAPK* pathways, were differentially expressed in PDAC compared with those in adjacent non-neoplastic tissues. However, neither the presence of *KRAS* mutation nor the extent of KRAS signaling dysregulation was associated with OS time. Among the KRAS downstream signaling pathway genes investigated, only *RAF1* expression was predictive of outcome. It is possible that the analysis of post-transcriptional and epigenetic factors associated with KRAS signaling may shed more light onto the molecular biology of PDAC.

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