Expression Patterns of Growth and Survival Genes with Prognostic Implications in Advanced Pancreatic Cancer

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Abstract. Aim: The aim of this study was to evaluate the mRNA expression pattern of growth- and survival-related genes and assess their prognostic significance in patients with advanced pancreatic cancer. Patients and Methods: In total, 98 patients were included in this retrospective translational research study and were evaluated for Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutational status, and v-akt murine thymoma viral oncogene homolog 1 (*AKT1*), *AKT* serine/threonine kinase 2 (*AKT2*), *AKT* serine/threonine kinase 3 (*AKT3*), cyclin *D1* (*CCND1*), epidermal growth factor receptor (*EGFR*), mitogen-activated protein kinase 1 (*MAPK1*), hepatocellular growth factor receptor (*MET*), avian myelomatosis viral oncogene homolog (*MYC*), nuclear factor kappa B subunit 1 (*NFKB1*), phosphatase and tensin homolog (*PTEN*) and mechanistic target of rapamycin (*FRAP1*) genes mRNA expression. Among these patients, 73 received first-line gemcitabine combined with erlotinib (*N*=57) or gefitinib (*N*=16). Results: *KRAS* mutation did not correlate with mRNA

gene expression. Unsupervised hierarchical clustering according to mRNA gene expression successfully distinguished four prognostically distinct groups of tumors. Overexpression of all genes was associated with best prognosis, while suppression or heterogeneous expression patterns of the examined genes were associated with expression patterns of growth- and survival-related genes, classifying pancreatic tumors into distinct groups with possibly different outcomes.

The epidermal growth factor receptor (*EGFR*) pathway has a major importance in pancreatic cancer(1). Erlotinib, a tyrosine kinase inhibitor (TKI) of *EGFR*, was tested in combination with gemcitabine as first-line treatment in a randomized trial of patients with advanced pancreatic cancer (2). The addition of erlotinib to gemcitabine demonstrated a modest but statistically significant survival benefit. Therefore, the identification of biomarkers for the selection of patient subgroups with a more meaningful benefit from erlotinib is mandatory. Multiple parameters, such as Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations, *EGFR* gene copy number alterations, protein expression and polymorphisms and phosphatase and tensin homolog (*PTEN*) protein expression have been examined in the populations of the PA.3 (2) and AIO-PK0104 (3) randomized trials. Unfortunately, none demonstrated predictive significance for benefit from *EGFR* modulation, whereas the results about their prognostic role were conflicting (4-8). Consequently, no specific molecular alteration has been found to predict response to *EGFR* inhibitors (9).

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Moreover, to our knowledge, no published study has examined the prognostic/predictive significance of growth and survival pathway gene-expression profiling in patients with pancreatic cancer treated with EGFR inhibitors. Among such genes, the most important is KRAS, which has been found to be mutated in approximately 70-90% of cases (5). *EGFR* and AKT serine/threonine kinase 2 (*AKT2*) amplification, and *PTEN* deletion are other important genetic aberrations. Increased AKT pathway activity has been found in up to 60% of pancreatic cancer tissues and cell lines. Murine leukemia viral oncogene (RAF)- mitogen-activated protein kinase (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase

(PI3K)-AKT pathway activation lead to up-regulation of cyclin D1 (*CCND1*) and avian myelomatosis viral oncogene homolog (*MYC*) transcription and activity and to stimulation of the nuclear factor kappa B subunit (NFkB) pathway (1, 10, 11). *MYC* overexpression is extremely common in pancreatic cancer tissues, while low amplification occurs in approximately 30% of cases (12). *CCND1* overexpression has been reported in 65-80% of pancreatic adenocarcinomas and has been associated with poor prognosis, whereas amplification was observed in 20% (13). The hepatocellular growth factor receptor (MET) receptor is overexpressed in pancreatic cancer, while the hepatocellular growth factor (HGF)/MET pathway is stimulated mostly by hypoxia. MET signaling is mediated mainly by the RAF-MAPK and PI3K-AKT pathways and seems to confer resistance to EGFR TKIs (10, 11). In view of the complexity of the accumulating preclinical data, we sought to retrospectively examine the correlation and significance of tissue mRNA levels of *AKT1*, *AKT2*, *AKT3*, *CCND1*, *EGFR*, *MAPK1*, *MET*, *MYC*, *NFKB1*, *PTEN* and mechanistic target of rapamycin (*FRAP1*) and *KRAS* mutational status in patients with pancreatic cancer treated with an EGFR TKI in the context of two prospective studies.

Patients and Methods

Patient population. Inclusion criteria were metastatic or locally advanced pancreatic cancer treated with first-line gemcitabine and erlotinib or gefitinib (prognostic cohort) or surgically resected pancreatic cancer treated with adjuvant gemcitabine or a fluoropyrimidine (molecular epidemiology cohort). In total, 98 patients with pancreatic adenocarcinoma treated from May 2001 to February 2011 in four Hellenic Cooperative Oncology Group (HeCOG) centers, available clinical data and formalin-fixed paraffin-embedded (FFPE) tumor tissue of sufficient RNA quality and quantity to obtain informative results for at least one biomarker were included in the study. Seventy-three had advanced unresectable and 25 surgically resected tumors. Gefitinib was administered in the context of a phase II single-arm trial of advanced pancreatic cancer (14), while erlotinib was given within an observational cohort study for advanced disease. The Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) flow chart of the study is shown in Figure 1.

Translational research study. This retrospective translational research study was approved by the HeCOG Protocol Review Committee and the Institutional Review Board of the Papageorgiou General Hospital (Approval number 649). The study complied with the REMARK recommendations for tumor marker prognostic studies using biological material (15). All patients signed an informed consent form for the provision of biological material for future research purposes.

Molecular methods. FFPE tumor tissue blocks from the 98 patients with pancreatic adenocarcinoma were processed for RNA extraction, following histological examination to estimate tumor tissue abundance. In order to enrich the molecular template with tumor transcripts, manual macrodissection was performed in cases with <50% tumor cell content; of note, such macrodissected samples also unavoidably contained tumor stroma. Upon macrodissection, tissue sections were transferred into a lysis buffer containing 500 µg/ml proteinase K for overnight lysis at 56°C. Total RNA was isolated from tissue lysates with TRIZOL-LS (Invitrogen/Life Technologies, Paisley, UK), according to the manufacturer's instructions. Following UV measurements, 4-5 µg of total RNA were reverse transcribed with random hexamers and SuperScript® III Reverse Transcriptase (all reagents from Invitrogen/Life Technologies, Carlsbad, CA, USA). Once cDNAs were normalized at 25 ng/µl, relative mRNA expression was assessed with quantitative polymerase chain reaction (qPCR) in an ABI7900HT system under default conditions. The following exon-spanning premade Taqman-MGB assays (Applied Biosystems/Life Technologies, Paisley, UK) were selected for the mRNA targets under investigation (data in parentheses refer to assay ID; Genbank reference; amplicon location; size): *AKT1* (Hs00178289_m1; NM_001014431.1, NM_001014432.1, NM_005163.2; exons 4-5, 5-6, 4-5; 66 bp), *AKT2* (Hs00609846_m1; NM_001626.3; exons 1-2; 129 bp), *AKT3* (Hs00178533_m1; NM_181690.1, NM_005465.3; exons 10-11; 100 bp), *CCND1* (Hs00765553_m1; NM_053056.2; exons 3-4; 57 bp), *EGFR* (Hs00193306_m1; NM_005228.3; exons 20-21; 69 bp), *MAPK1* (Hs01046830_m1; NM_138957.2, NM_002745.4; exons 6-7; 74 bp), *MET* (Hs00179845_m1; NM_001127500.1, NM_000245.2; exons 10-11; 81 bp), *MYC* (Hs00153408_m1; NM_002467.4; exons 2-3; 107 bp), *NFKB1* (Hs00231653_m1; NM_001165412.1, NM_003998.3; exons 19-20; 73 bp), *PTEN* (Hs02621230_s1; NM_000314.4; exons 9-9; 135 bp) and *FRAP1* (Hs00234508_m1; NM_004958.3; exons 20-21; 103 bp).

Samples were processed in 10 µl reactions (50 ng cDNA/reaction) with TaqMan® Universal PCR Master Mix and run in duplicates in 384-well plates. As a positive control, a commercially available reference RNA (TaqMan® Control Total RNA, cat. no 4307281, Applied Biosystems/Life Technologies) was included in each run. Moreover, a Taqman-MGB expression assay targeting β-glucuronidase (*GUSB*) mRNA (Hs00939627_m1; NM_000181.3; exons 8-9; 96 bp) was used as an endogenous control and for the normalization of cycle threshold (CT) values. Relative quantification (RQ) was assessed in a linear mode as (40-ΔCT), whereby ΔCT=(avg CT *target*) - (avg CT *GUSB*). Exclusion criteria from RQ analysis were *GUSB* CT values higher than 36 and duplicate ΔΔCT of >0.8 in the same run, whereas PCR assay stability was evaluated among runs with the reference RNA.

Methods of DNA extraction and KRAS mutation assessment. DNA extraction was performed from macrodissected tissue fragments as above, with the QIA amp DNA Mini Kit (Qiagen, Hilden,

Figure 1. REMARK flow chart.

Germany), according to the manufacturer's instructions. *KRAS* mutations (exons 2, 3, 4) were assessed with dd-sequencing on nested PCR amplicons. Primers were located in adjacent introns spanning the entire coding regions of interest and are available upon request. Nested primers were coupled with universal M13 forward and reverse primers at the 5'-end. Sense and antisense sequencing was performed using M13 forward and reverse primers in 10 µl reactions with the Big Dye Terminator kit v.1.1 (Applied Biosystems/Life Technologies). Products were visualized upon capillary electrophoresis in an ABI3130XL genetic analyzer, base called and further analysed with Sequencing Analysis version 5.2 software (Applied Biosystems).

Statistical analysis. Comparisons of categorical with continuous variables were made using the Mann–Whitney or the Kruskal–Wallis tests, while the Fisher's exact test was used for testing associations between categorical variables. Pearson's correlation coefficients were used to assess correlations among continuous

variables. Separation of the continuous data for markers into high and low expression groups was performed using the median cut-off.

For the patients with advanced cancer, progression-free survival (PFS) was measured from the time of initiation of first-line treatment until verified disease progression, death or last contact (whichever occurred first), while overall survival (OS) was measured from the time of initiation of first-line treatment until death from any cause or date of last contact. For the adjuvantly treated patients, disease-free survival (DFS) and OS were measured from the date of initiation of adjuvant treatment. Time-to-event distributions were estimated using Kaplan–Meier curves, while log-rank tests and univariate Cox analyses were used for assessing differences statistically and reporting hazard ratios, respectively.

Unsupervised hierarchical clustering (using Ward's minimum variance method) was conducted for the mRNA markers in order to identify distinct groups correlating with prognosis. Multivariate analysis was not performed because the sample size was too small. All tests were two-sided. The statistical analysis complied with the reporting recommendations for tumor marker prognostic studies (15) and was performed using SAS software (SAS for Windows, version 9.3; SAS Institute Inc., Cary, NC, USA).

Results

Basic patient and disease characteristics. Basic patient and disease characteristics are depicted in Table I. Among those with advanced pancreatic cancer, 53 patients (72.6%) were initially diagnosed with metastatic disease, while the rest with earlier stages. Fifty-seven patients were treated with gemcitabine-erlotinib and 16 with gemcitabine-gefitinib. The median number of cycles was 6 for patients with advanced disease and 6 for those treated with adjuvant chemotherapy (range=1-16 and 1-27, respectively).

Response to treatment and survival. Survival data were available for 15 patients with resected disease and for 70 with advanced disease. The median follow-up for adjuvantly treated patients was 40.5 months, while for those with advanced disease, it was 6.1 months (range=2.6-71.8 and 0.5-32.2, respectively). By the last date of follow-up, 11 out of the 15 patients treated with adjuvant chemotherapy had experienced disease relapse, with a median DFS of 15 months and 1-year DFS of 60%. Nine patients had died, with a median OS of 23 months and 1-year OS of 73.3%. Among the 70 patients with advanced disease, in 61 disease had progressed and 56 had died, with a median PFS and OS of 5 and 7 months, respectively, and a 1-year PFS and OS of 7.6% and 30%, respectively. Only four patients responded to treatment (response rate=5.4%).

mRNA expression studies. RQ values of gene expression were obtained for 98 patients in total, 93 patients for *AKT1*, 85 for *AKT2*, 78 for *AKT3*, 83 for *CCND1*, 86 for *EGFR*, 85 for *MAPK1*, 87 for *MET*, 82 for *MYC*, 87 for *NFKB1*, 87 for *PTEN* and 90 for *FRAP1*. Figure 1 shows the REMARK flow chart of the present study and the numbers

Table I. Patient and disease characteristics.

		Group	
		Adjuvant	Advanced
Patients	N	25	73
Age, years	Mean (SD)	62.2 (10.8)	63.4 (11)
	Min-Max	42-77	36-80
Karnofsky PS	Median	90%	80%
Gender, n (%)	Female	7 (28)	27 (37)
	Male	18 (72)	46 (63)
Histological grade, n (%)	G1, G2	13 (52)	35 (48)
	G3, G4, GX	12 (48)	23 (32)
	Not reported		15 (21)
TNM stage at diagnosis, n (%)	I	3 (12)	1 (1.4)
	II	19 (76)	10 (14)
	III	2 (8)	6 (8)
	IV	0 (0)	53 (73)
	Not reported	1 (4)	3 (4)

PS: Performance status.

that correspond to the 98-patient cohort. The majority of available tissues were from the pancreatic primary site (N=77, 78.6%), seven (7.1%) were from regional lymph nodes and 13 (13.3%) from distant metastatic sites, while in one (1.0%), the site of origin was not reported. Among 73 patients with advanced disease treated with gemcitabine and an EGFR TKI, nine (12.3%) had tissue available only from prior surgery, while tissue samples were available from biopsies in remaining cases. RQ gene expression levels in patients with advanced disease are shown in Figure 2.

Correlations. Significant correlations ($p<0.0001$) were found most frequently with *MAPK1*, *NFKB1* and *CCND1*. The strongest correlations (correlation coefficient ≥ 0.6) were observed for *MAPK1* with *NFKB1*, and for *MAPK1* with *PTEN*, as shown in Table II.

Correlations between marker RQ levels and age, gender and histological grade were also tested. Males had relatively higher levels of mRNA expression of *CCND1* (median RQ: 44.2 vs. 43.6, $p=0.027$), EGFR (median RQ: 40.4 vs. 40.0, $p=0.042$) and *MET* (median RQ: 41.3 vs. 40.5, $p=0.045$). In addition, patients ≥ 60 years old had higher levels of mRNA expression of *MAPK1* (median RQ: 41.7 vs. 41.4, $p=0.046$), *MET* (median RQ: 41.5 vs. 40.5, $p=0.015$), *NFKB1* (median RQ: 40.3 vs. 39.9, $p=0.013$) and *PTEN* (median RQ: 40.2 vs. 39.5, $p=0.011$).

KRAS mutations and their correlation with gene expression. *KRAS* mutations were evaluated in 80 patients, 58 with advanced- and 22 with early-stage disease. Among them,

Table II. Significant correlations (all $p<0.0001$) between relative quantification (RQ) values for expression of the genes studied.

Variable	With variable	N	Correlation coefficient	Lower 95% confidence limit	Upper 95% confidence limit
<i>AKT1</i>	<i>CCND1</i>	95	0.50	0.33	0.64
	<i>MAPK1</i>	97	0.59	0.44	0.70
	<i>NFKB1</i>	98	0.53	0.37	0.66
<i>CCND1</i>	<i>EGFR</i>	91	0.50	0.33	0.64
	<i>MAPK1</i>	91	0.57	0.41	0.69
	<i>MET</i>	93	0.50	0.33	0.64
	<i>NFKB1</i>	92	0.56	0.40	0.68
	<i>PTEN</i>	93	0.56	0.40	0.68
<i>EGFR</i>	<i>MAPK1</i>	94	0.44	0.25	0.59
	<i>NFKB1</i>	95	0.46	0.28	0.60
<i>MAPK1</i>	<i>MET</i>	97	0.44	0.26	0.58
	<i>MYC</i>	91	0.45	0.27	0.60
	<i>NFKB1</i>	94	0.78	0.68	0.84
	<i>PTEN</i>	95	0.61	0.47	0.72
<i>MET</i>	<i>NFKB1</i>	96	0.46	0.28	0.60
<i>MYC</i>	<i>NFKB1</i>	92	0.59	0.44	0.71
	<i>PTEN</i>	93	0.40	0.21	0.55
<i>NFKB1</i>	<i>PTEN</i>	96	0.68	0.55	0.77

The strongest Pearson's correlation coefficients (>0.5) are shown in bold.

KRAS mutations were detected in 56 patients (70%), 38 (65.5%) with advanced-stage and 18 (81.8%) with early-stage. The types of *KRAS* mutations and their frequencies are reported in Table III. *KRAS* mutations were not correlated with mRNA RQ values of any of the genes under study.

Figure 2. Boxplot for the relative quantification (RQ) gene expression levels (patients with advanced cancer stage).

Prognostic factor analysis. Prognostic factor analysis was only performed for patients with advanced pancreatic cancer because of the low number of patients treated with adjuvant chemotherapy with available survival data. Univariate analysis of prognostic factors for advanced disease showed only a marginally significant favorable effect of high *NFKB1* mRNA RQ level on PFS (7 vs. 4 months, $p=0.0495$). *AKT3* levels above the median were associated with marginally significantly higher response rate (14.3% vs. 0.0%, $p=0.045$). Interestingly, *KRAS* mutational status did not significantly influence PFS or OS, nor the objective response rate.

Hierarchical clustering analysis. In patients with advanced pancreatic cancer, gene-expression signatures were created by clustering, a statistical method that classifies tumors into groups, according to their gene expression RQ values, in order to achieve the highest similarity within groups and the highest distinction between groups. Clustering is not influenced by parameters, such as response or survival. Clustering into 2, 3 or 4 groups was attempted and as expected, clustering into four groups more successfully distinguished tumors into distinct clusters (Figure 3). Clusters 1 and 2 were characterized by predominantly high or low gene-expression levels, respectively. In cluster 1, gene expression followed a more 'normal' distribution, with relatively high *EGFR* mRNA expression. Cluster 2 was

Table III. Types of Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations and their frequencies.

	Total		Group			
			Adjuvant		Advanced	
	N	%	N	%	N	%
<i>KRAS</i>						
Mutated	56	70.0	18	81.8	38	65.5
WT	24	30.0	4	18.2	20	34.5
Total	80	100.0	22	100.0	58	100.0
<i>KRAS</i> mutation type						
p.G12C	10	17.9	2	11.1	8	21.1
p.G12D	28	50.0	8	44.4	20	52.6
p.G12R	4	7.1	0.0	0.0	4	10.5
p.G12V	14	25.0	8	44.4	6	15.8
Total	56	100.0	18	100.0	38	100.0

WT: Wild-type.

characterized by low expression of the majority of markers and moderate expression of *AKT2* and *MET*. In cluster 3, the studied genes had extremely low mRNA expression. The receptors *EGFR* and *MET*, the mediators *AKT1* and *MAPK1*, the regulatory molecule *NFKB1*, the inhibitory factor *PTEN* and the downstream effectors *MYC* and *CCND1*, all

Figure 3. Unsupervised hierarchical clustering into four groups of relative quantification (RQ) values. Heatmap of the four clusters: Cluster 1, red (n=30); Cluster 2, orange (n=11); Cluster 3, blue (n=3); Cluster 4, green (n=2). High RQ values are red and low values blue.

Table IV. Median overall (OS) and progression-free (PFS) survival for each cluster of patients.

Standardized clustering	N	Median OS (months)	95% CI	Median PFS (months)	95% CI
Cluster 1	30	8.3	5.2-13.8	5.7	3.4-7.5
Cluster 2	11	6.5	1.4-18.7	4.1	1.4-8.1
Cluster 3	3	6.9	4.0-9.8	4.0	1.4-5.1
Cluster 4	2	2.8	2.6-2.9	1.9	1.1-2.6

CI: Confidence interval.

Figure 4. Overall (OS) (a) and progression-free (PFS) (b) survival for the four patient clusters.

demonstrated markedly low expression. Cluster 4 included only two cases, which may represent outliers or a true biologically separate subset. The predominant common feature of these two cases was the distinct gene-expression pattern, with elevated expression of *MAPK1*, *PTEN*, *NFKB1*, *FRAP1*, *AKT3* and *MET* and relatively lower mRNA RQ levels of *AKT2*, *MYC* and *CCND1*.

The median OS and PFS were calculated for each cluster (Table IV). Interestingly, patients with tumors expressing predominantly high transcript levels of the genes examined (cluster 1) seemed to have the best OS and PFS (log-rank, $p=0.009$ and $p=0.035$, respectively), although the number of patients was too low to draw definitive conclusions (Figure 4).

Discussion

Pancreatic adenocarcinoma is an aggressive malignancy, relatively resistant to antineoplastic treatment. Until now, it has been approached as a single disease entity and treatment decisions were based on clinical criteria only, such as stage, age and performance status. Recent research has focused on subclassifying tumors into groups according to prognostic/predictive biomarkers in order to better understand disease biology and ultimately improve treatment, with significantly heterogeneous results (16-19). Numerous prognostic biomarkers have been described but a significant proportion of them have been studied in pancreatectomy specimens from patients mostly treated with postoperative chemotherapy (17, 18, 20, 21). In contrast, translational research studies in patients treated with newer agents, such as EGFR inhibitors, are scarce and only a small number of biomarkers has been tested.

In the present study, about 75% of the patients were treated with gemcitabine plus an EGFR TKI (erlotinib or gefitinib) for advanced disease. The rest underwent resection of localized disease and received adjuvant chemotherapy, mostly gemcitabine monotherapy. This adjuvant cohort was included in the study in order to enrich our molecular epidemiology data regarding the expression pattern of multiple genes of the growth and survival pathways.

Some of these genes are known to be primarily affected, such as *EGFR*, *AKT2*, *CCND1* and *MYC* by amplification, and *PTEN* by deletion. Although most of the studied biomarkers had demonstrated prognostic significance in previous studies, this was not the case in our study. Moreover, in contrast to the literature (22, 23), *NFKB1* was shown to have marginally favorable prognostic significance (21). This could easily be attributed to the small size of the patient population, although two significant factors should be considered. The first is that these biomarkers had almost exclusively been previously studied by immunohistochemistry (1, 11-13, 22-24), and not mRNA expression. The second is that most of the factors had never been studied in patients treated with EGFR inhibitors

(1, 11-13). In our study, the absence of a control group of patients with advanced pancreatic cancer not receiving EGFR inhibitors with chemotherapy makes it impossible to dissect potential prognostic from predictive significance. It could be hypothesized that some biomarkers might have adverse prognostic and favorable predictive utility in the presence of erlotinib or gefitinib, the latter diluting the former in the absence of a control group.

The prognostic *versus* predictive significance of any given biomarker can only be investigated in a randomized trial. Although the translational research results of the PA.3 and AIO-PK0104 studies were negative for the predictive and conflicting for the prognostic significance of a limited number of biomarkers (4-6), the existence of significant correlations in their expression levels, as shown in our study, probably reflects the complexity of tumor biology. This could explain the negative results and may only be overcome by studying genetic signatures rather than single molecular alterations.

In a study of pancreatic adenocarcinoma xenograft models, Jimeno *et al.* studied mRNA expression, mutations, copy number changes and protein expression of multiple EGFR pathway genes. Interestingly, neither genetic alterations nor immunohistochemistry predicted sensitivity to EGFR inhibitors. On the other hand, global increase in the expression and activation of the EGFR pathway-related genes accurately predicted drug efficacy (22).

In our study, multigene expression was able to discriminate patients with advanced pancreatic tumors into four clusters, with similar expression patterns within-clusters for most of the studied genes. Cluster 1 was characterized by a homogeneously high gene expression pattern. This globally increased expression may be a surrogate marker of an EGFR pathway addiction of the tumor and a possible sensitivity to EGFR inhibitors. In contrast, clusters 2 and 3 demonstrated moderate and extremely low expression of most of the genes, respectively. The marked down-regulation of most of the genes in cluster 3 may reflect dependence on other growth and survival pathways. Finally, cluster 4 showed a heterogeneous gene expression pattern, with up-regulation of genes, such as *MAPK1*, *NFKB1*, *PTEN*, *FRAP1* and *MET*, not known to be commonly altered in pancreatic cancer. Their high relative expression may result from other genetic alterations that influence MET expression, as well as from the PI3K-AKT-mTOR and MAPK-NFkB pathways.

Survival analysis showed that patients of clusters 1 and 2 had statistically longer OS and PFS than those of clusters 3 and 4, although the small sample size does not allow for any definitive conclusion to be drawn. The rarity of responses precluded any analysis for prediction of objective tumor regression. However, considering the observations of Jimeno *et al.* (22), global overexpression of the present gene set may be associated with favorable prognosis due to a relatively better responsiveness to EGFR inhibitors. Notably, Collisson *et al.*

found that their favorable prognosis gene-expression signature was also associated with higher sensitivity to erlotinib but lower sensitivity to gemcitabine, although the EGFR pathway family was underrepresented in their gene set (16).

Conclusion

In the current study, we report on the emergence of gene-expression signatures with potential prognostic significance in a cohort of patients with advanced pancreatic cancer homogeneously managed with the combination of gemcitabine and an EGFR TKI. This study supports the concept that gene-expression signatures may better reflect the complexity of pancreatic adenocarcinoma biology and possibly offer useful prognostic or even predictive information. Nevertheless, these results should be confirmed and validated in larger independent cohorts.

Conflicts of Interest

The Authors declare that they have no competing interests. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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