

## High Expression of *HIF1a* Is a Predictor of Clinical Outcome in Patients with Pancreatic Ductal Adenocarcinomas and Correlated to *PDGFA*, *VEGF*, and *bFGF*

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### Abstract

**PURPOSE:** Pancreatic cancer still has one of the worst prognoses in gastrointestinal cancers with a 5-year survival rate of 5%, making it necessary to find markers or gene sets that would further classify patients into different risk categories and thus allow more individually adapted multimodality treatment regimens. In this study, we investigated the prognostic values of *HIF1a*, *bFGF*, *VEGF*, and *PDGFA* gene expressions as well as their interrelationships. **EXPERIMENTAL DESIGN:** Formalin-fixed paraffin-embedded tissue samples were obtained from 41 patients with pancreatic adenocarcinoma (age, 65; range, 34–85 years). After laser capture microdissection, direct quantitative real-time reverse transcription–polymerase chain reaction assays were performed in triplicates to determine *HIF1a*, *PDGFA*, *VEGF*, and *bFGF* gene expression levels. Multivariate Cox proportional hazards regression analysis was used to assess the impact of *HIF1a* gene expression on prognosis. **RESULTS:** *HIF1a* was significantly correlated to every gene we tested: *bFGF* ( $P = .04$ ), *VEGF* ( $P = .02$ ), and *PDGFA* ( $P = .03$ ). Tumor size,  $P = .04$ , and high *HIF1a* mRNA expression (cutoff, 75th percentile) had a significant impact on survival,  $P = .009$  (overall model fit,  $P = .02$ ). High *HIF1a* expression had a sensitivity of 87.1% and a specificity of 55.6% for the diagnosis short (<6 months) versus long (6–60 months) survival. **CONCLUSIONS:** Measuring *PDGFA*, *bFGF*, and *HIF1a* expression may contribute to a better understanding of the prognosis of patients with pancreatic cancer and may even play a crucial role for the distribution of patients to multimodal therapeutic regimens. Larger studies including patients treated with actual chemotherapeutics seem to be warranted.

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### Introduction

Although rising for over 30 years, mortality rates of adenocarcinoma of the pancreas in Europe have leveled off in the last 10 to 15 years and have even decreased approximately 4% in the United States [1,2]. Nonetheless, pancreatic cancer still has one of the worst prognoses in gastrointestinal cancers, with a 5-year survival rate of 5%. Due to the late symptoms of pancreatic cancer and therefore often late diagnosis, only 10% to 20% of the patients are eligible for complete resection with curative intention, making it necessary to find

Abbreviations: HIF1a, hypoxia-inducible factor 1 alpha; PDGFA, platelet-derived growth factor alpha; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; FFPE, formalin-fixed paraffin-embedded

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markers or gene sets that would further classify patients into different risk categories and thus allow more individually adapted multimodality treatment regimens [3].

Several good candidate prognostic markers for pancreatic adenocarcinoma have been identified by previous work. Hypoxia-inducible factor 1 alpha (HIF1 $\alpha$ ) has been shown to correlate with an unfavorable prognosis in many cancers and is known to regulate some genes in the angiogenesis pathway [4]. Recently, Sun et al. [5] and Tao et al. [6] showed with immunohistochemical methods that HIF1 $\alpha$  not only has a strong impact on the prognosis of patients with pancreatic ductal adenocarcinoma but is also correlated with vascular endothelial growth factor (VEGF) expression. Studies on human pancreatic cancer cells revealed that hypoxia and paracrine secretion of insulin induced HIF1 $\alpha$  expression, which in turn led to stimulated glycolysis, cell proliferation, and VEGF secretion [7]. HIF1 $\alpha$  binds to the promoter region of VEGF and apparently thereby increases the expression of VEGF under hypoxic conditions [8]. VEGF is not only a key factor in the angiogenesis pathway but also a molecular drug target for several U.S. Food and Drug Administration–approved chemotherapeutics [9]. Platelet-derived growth factor alpha (PDGFA) has recently been discussed as a potential drug target in pancreatic cancer [10]. It has to be further investigated in what respect HIF1 $\alpha$  and PDGFA are coexpressed in pancreatic cancer. The expression of basic fibroblast growth factor (bFGF) is known to have a strong association with the prognosis of patients with pancreatic cancer [11]. Some study groups showed a correlation between bFGF and HIF1 $\alpha$  expression in patients with breast and non–small cell lung cancer [12,13]. However, this association is, like that of PDGFA and HIF1 $\alpha$ , little studied in pancreatic cancer.

In this study, we investigated the prognostic values of HIF1 $\alpha$ , bFGF, VEGF, and PDGFA gene expressions as well as their interrelationships in pancreatic cancer. We measured the mRNA expression levels of these genes with quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) in formalin-fixed paraffin-embedded (FFPE) tissue samples of pancreatic carcinoma. We then further analyzed the previously mentioned genes and their correlation with clinical and histopathologic variables such as tumor size (diameter/volume), primary tumor stage [pT], based on the International Union Against Cancer (UICC), 1997, regional lymph node metastasis, grading, and especially the survival time.

## Materials and Methods

### Study Population, Demographic Data, and Staging Procedures

Formalin-fixed paraffin-embedded samples were obtained from 41 patients with pancreatic adenocarcinoma with a median age of 65 years (range, 34–85 years) at the time of operation who were scheduled for primary surgical resection. None of the patients had received neoadjuvant or adjuvant radio-/chemotherapy. All patients were treated at the University hospital of Cologne, North Rhine-Westphalia, Germany, between December 1999 and July 2004. Demographic, clinical, and histopathologic parameters are shown in Table 1. Informed consent was obtained from each patient in accordance with the requirements of our institution's board of ethics. TNM staging was performed according to the criteria of the UICC [14].

### Microdissection

After a review of representative hematoxylin and eosin–stained slides of the FFPE blocks by a pathologist to estimate the tumor load

**Table 1.** Patient Characteristics (N = 41).

Parameter	n (%)
Median age: 65 years (range, 34–85 years)	
Sex	
Male	23 (56.1)
Female	18 (43.9)
Histologic diagnosis	
Adenocarcinoma	41 (100)
pT category	
pT1	1 (2.4)
pT2	6 (14.6)
pT3	32 (78.0)
pT4	2 (4.9)
pN category	
N –	7 (17.1)
N +	33 (80.5)
Not evaluated	1 (2.4)
c/pM category	
c/pM0	41 (100)
Grading	
G2	22 (53.7)
G3	19 (46.3)
Residual tumor category	
R0	41 (100)
Tumor size (diameter; cm)	
Minimum	1
Maximum	7
Range	6

UICC 1997 Tumor-Node-Metastasis (pTNM) Pathological Classification: pT indicates primary tumor; pN, regional lymph node metastasis; c/pM, distant metastasis; G, grade of differentiation; R, residual tumor category.

per sample, section slides of 10- $\mu$ m thickness were obtained for laser-captured microdissection (P.A.L.M. Microlaser Technologies AG, Munich, Germany). All tumor slides were prepared as described extensively by Vallbohmer et al. [15].

### Isolation of RNA and cDNA Synthesis

The isolation of RNA from tumor tissue isolated by the microdissection was performed in accordance with a patented procedure at Response Genetics Inc (Los Angeles, CA; U.S. Patent No. 6248,535). The cDNA preparation steps were accomplished as described previously [16].

### Quantitative Real-Time Polymerase Chain Reaction

To quantify HIF1 $\alpha$ , PDGFA, VEGF, and bFGF mRNA expression levels, we used an endogenous reference gene ( $\beta$ -actin) and our gene set on a method based on real-time fluorescence detection of amplified cDNA (ABI PRISM 7900 Sequence Detection System [TaqMan]; Perkin-Elmer Applied Biosystems, Foster City, CA). The RT-PCR was implemented as previously described by Kuramochi et al. [17]. All genes were run on all samples in triplicates. The detection of amplified cDNA results in a cycle threshold ( $C_t$ ) value that is inversely proportional to the amount of cDNA. The higher the ensuing  $C_t$  value, the more PCR cycles were necessary to attain detection limit, which means less cDNA. Colon, liver, and St. Universal Mix RNA (Stratagene, La Jolla, CA) were used as control calibrators on each plate. All primers were selected using the Gene Express software (Applied Biosystems) but were adapted to the needs of RNA/cDNA as extracted out of the paraffin-embedded tissue. All primers were validated before use analogical to the described method of Schneider et al. [18]. All results are expressed as ratios between two absolute measurements (gene of interest/endogenous reference gene)

to account for loading differences. We used a log transformation before statistical analysis including a multiplier that accounts the average  $C_t$  values maintained for each gene during the validation process on the calibrators and therefore allows comparing samples that were run on different RT-PCR well plates.

### Statistical Analysis

The correlation among gene expression levels, those and clinicopathologic parameters were tested with Spearman test for bivariate correlations. Each gene was tested with the Kaplan–Meier method to estimate overall survival and relapse-free survival. Differences in survival between the high- and low-expression groups were analyzed with the log rank test. To evaluate independent prognostic factors associated with survival, multivariate Cox proportional hazards regression analysis with stepwise selection was used, with the gene set, tumor stage, tumor size (diameter/volume), and histologic characteristics (grading) as covariates. A data mining technique provided by the SAS Institute was used to split gene expression in high- and low-level groups based on a platform that recursively partitions data according to a relationship between the  $X$  and  $Y$  values, creating a tree of partitions (recursive descent partition analysis). By searching all possible cuts, it finds a set of cut points of  $X$  values (Gene Expression) that best predict the  $Y$  value (Survival Time). These data splits are done, recursively forming a tree of decision rules until the desired fit is reached; the most significant split is determined by the largest likelihood ratio chi-square statistic. In either case, the split is chosen to maximize the difference in the responses between the two branches of the split. This method was previously used by Lu et al. [19]. We used receiver operating characteristic (ROC) curve analysis to test the ability of the chosen cutoffs to discrimi-

nate short survivors (<6 months) from long survivors (6 months to 5 years). The level of significance was set to  $P < .05$ . All  $P$  values reported were based on two-sided tests.

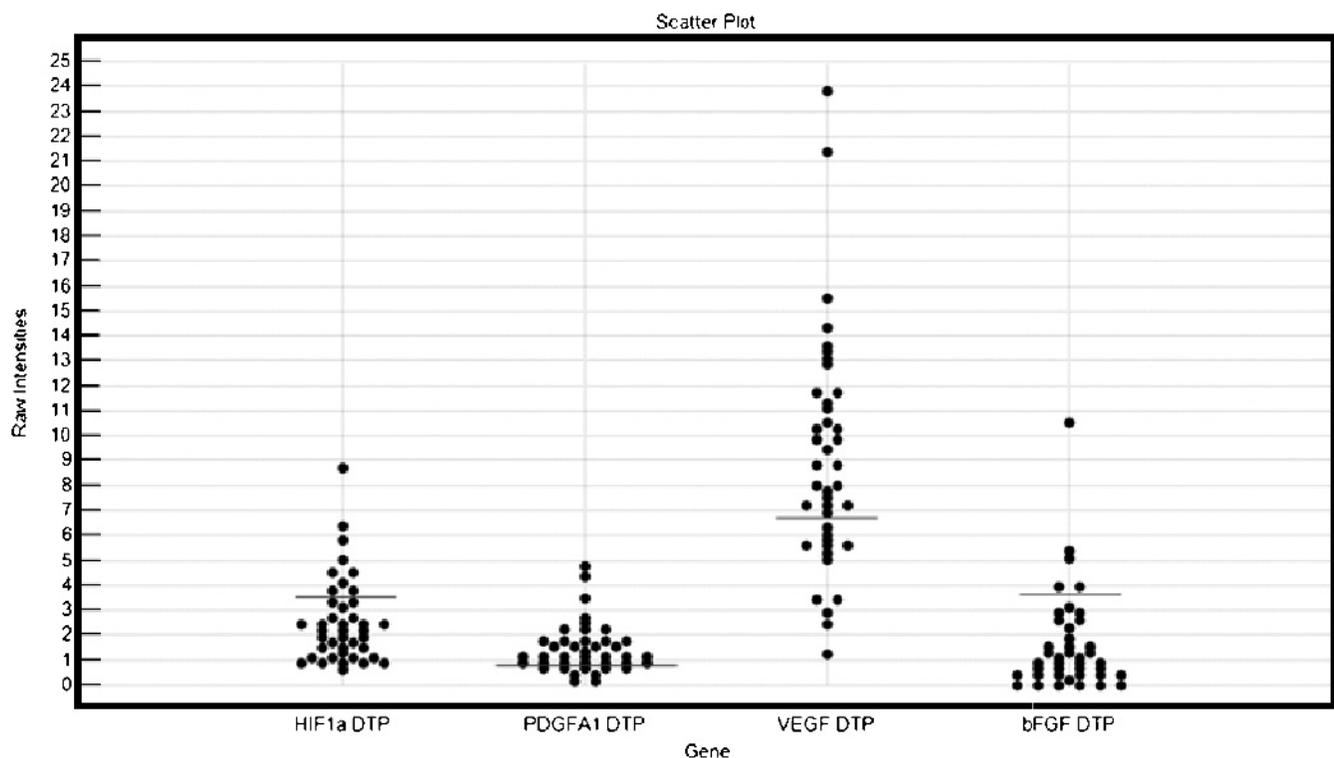
All statistical tests were performed using the Statistical Package for the Social Sciences for Windows (Version 16.0; Chicago, IL) and the JMP 7.0 Software (SAS, Cary, NC).

### Results

The distribution of the log-transformed  $\Delta C_t$  values is shown in Figure 1.

#### Spearman Test for Bivariate Correlations

Spearman test on the log-transformed  $\Delta C_t$  values showed significant correlations between some of the gene expressions. *HIF1a* was correlated to every gene we tested. *HIF1a* was significantly correlated to *bFGF* at  $P = .04$  ( $P < .05$ ), it was associated to the *VEGF* gene expression with a significance of  $P = .02$  ( $P < .05$ ) and to *PDGFA* at a level of significance of  $P = .03$  ( $P < .05$ ). *VEGF* gene expression and *PDGFA* expression levels were associated with a significance of  $P = .02$  ( $P < .05$ ). We performed partition analysis of *VEGF* mRNA expression based on the existence of lymph node metastasis, thereby obtaining two groups with high *versus* low expression profiles. This grouping showed a significant correlation to lymph node metastasis with  $P = .04$  ( $P < .05$ ), thus implicating that a high expression of *VEGF* is linked to a higher likelihood of lymph node metastasis. Using the same method for the other used genes, no significant correlation appeared. With a significance level of  $P = .01$  ( $P < .05$ ), a high *VEGF* mRNA level was also connected to the size (in diameter) of the resected pancreatic adenocarcinoma.



**Figure 1.** Scatterplot of the log-transformed  $\Delta C_t$  values for the studied genes. The lines represent the cutoff values based on recursive descent partition analysis.

### Partition Tree Analysis of Genes Based on Survival Time

Using the survival (in days) as the factor to perform partition analysis on the chosen gene set, *bFGF* showed up as the most significant divisor (with the highest log rank). The next in line were first *HIF1a*, then *PDGFA*, and then *VEGF*. The cutoff value for *HIF1a* was 3.9 (the 75th percentile) leaving the high-expression group of 11 patients with a mean of 4.8 (range, 3.9–8.7) and the low-expression group consisting of 30 patients with a mean of 3.2 (range, 0.6–3.8). The groupings, respectively, the cutoffs for the other genes are indicated in Figure 1.

### Survival Analysis Using the Kaplan–Meier Method

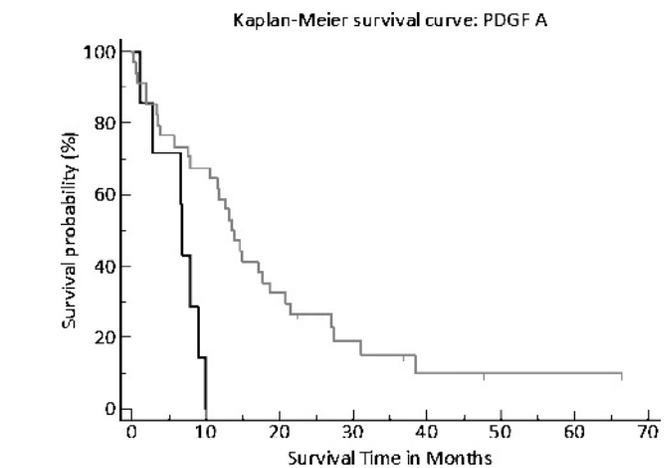
The groupings obtained by the recursive descent partition analysis were used for each gene to assign survival with Kaplan–Meier log rank analysis. The level of significance for *bFGF* was  $P = .01$  (Figure 2) and for *PDGFA*,  $P = .003$  (Figure 3). The  $P$  value of *HIF1a* only showed a trend to significance.

### Multivariate Cox Proportional Hazards Regression Analysis

We put all clinical and histopathologic parameters along with the measured gene expressions in a stepwise multivariate Cox proportional hazards regression model. The overall model fit had a significance level of  $P = .02$ . There were two factors that had a significant impact on the survival time in this patient cohort, namely, tumor size,  $P = .04$ , and a high *HIF1a* mRNA expression (75th percentile cutoff), with a significance level of  $P = .009$  (Figure 4).

### Receiver Operating Characteristic

The 75th percentile cutoff of the *HIF1a* mRNA expression showed a sensitivity (true-positive rate) of 87.1% and a specificity (true-negative rate) of 55.6% for the diagnosis short (<6 months) versus long (6 months to 5 years) survival. The area under the curve was 0.713 (confidence interval, 0.549–0.845) with a significance level of  $P = .043$ . The positive likelihood ratio (true-positive rate/



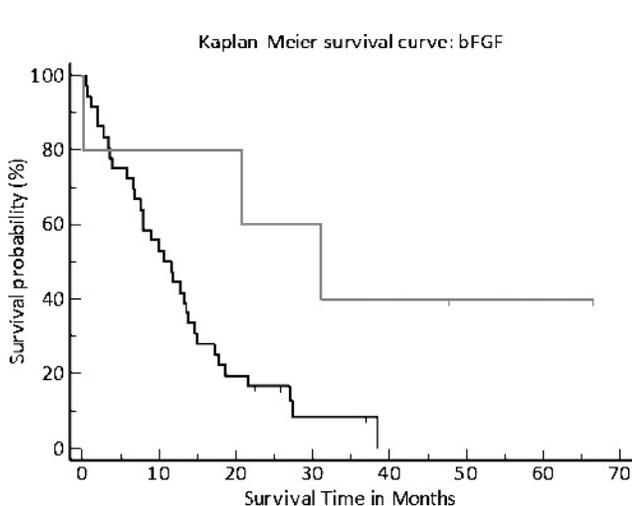
**Figure 3.** Kaplan–Meier plot, estimating overall survival and relapse-free survival. Differences in survival between the high and the low *PDGFA* expression groups were analyzed with the log rank test.

false-positive rate) was 1.96, and the negative likelihood ratio (false-negative rate/true-negative rate) was 0.23.

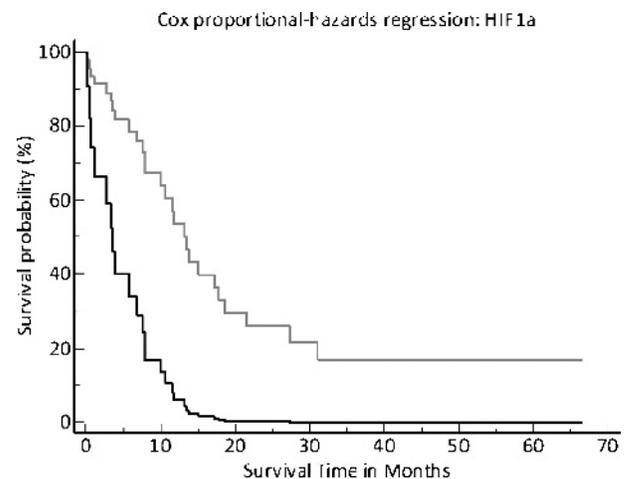
### Discussion

In this study, we determined the gene expressions of *HIF1a*, *VEGF*, *bFGF*, and *PDGFA* in the FFPE samples of pancreatic cancer patients who did not receive any chemotherapy. By using laser capture microdissection to isolate tumor tissue from the clinical specimens along with quantitative RT-PCR, we hoped to achieve a more precise characterization of the associations of these gene expressions with each other and with patients' prognosis than was previously available. By multivariate Cox regression analysis, *HIF1a* gene expression showed the most significant impact on prognosis in our patient cohort ( $P = .009$ ).

It has to be mentioned that *HIF1a* is known to undergo a rapid posttranscriptional degradation under normoxic conditions by the



**Figure 2.** Kaplan–Meier plot, estimating overall survival and relapse-free survival. Differences in survival between the high and the low *bFGF* expression groups were analyzed with the log rank test.



**Figure 4.** Survival plot from multivariate Cox regression analysis estimating overall survival and relapse-free survival. The upper light gray line represents the patients with a *HIF1a* expression of lower than the 75th percentile.

ubiquitin–proteasome system, which is restricted by an oxygen-dependent degradation domain within HIF-1, but under hypoxic conditions, the accumulation of *HIF1a* involves stabilization of the protein [20]. For several genes, such as *thymidylate synthase* and *dihydropyrimidine dehydrogenase*, there have been studies that provided information on a generally close linear correlation between the expression of mRNA and the protein expression [21,22]. Although the regulation of *HIF1a* RNA expression seems to respond rapidly to the oxygen concentrations in the cell [23], meaning that the mechanisms of *HIF1a* regulation are transcriptional as well as posttranscriptional, it is controversially discussed whether these mechanisms correlate well to each other [24]. In this study, we show that the determination of quantitative levels of gene expressions may be valuable and important in itself, regardless of whether it matches the protein or not. Although there is posttranscriptional regulation of the *HIF1a* subunit, an approach to use mRNA expression for assessing the prognosis of patients with pancreatic ductal adenocarcinoma seems to be feasible.

The results of this pilot study also show a strong correlation of *HIF1a* to *VEGF* and underline the meaning of *HIF1a* for the angiogenesis and its most prominent marker *VEGF* on pancreatic cancer. Although we could not verify the results of Sun et al. [5] regarding the meaning of *VEGF* for the survival of the patients, we were also able to show that a high *VEGF* expression significantly correlated to lymph node metastasis ( $P = .04$ ) and tumor size ( $P = .01$ ).

The heparin-binding *bFGF* is one of the more frequently described genes in pancreatic cancer. The association of *HIF1a* and *bFGF* has recently been shown in some cancers. Bos et al. [12] investigated 45 samples of invasive breast cancer with immunohistochemistry. They were able to show a significant association between *HIF1a* and *bFGF* but could not show this for *VEGF* and *HIF1a*. Also, a different member of the PDGF family than we studied had a significant connection to *HIF1a* expression. Giatromanolaki et al. [13] examined 120 samples of patients with non–small cell lung cancer with monoclonal antibodies on FFPE samples, assessing the relationship between *HIF1a*, *bFGF*, *VEGF*, and *PD-ECGF*. In their patient cohort, the association between the expression of these proteins was significant. A literature search disclosed no studies focusing on the relationship between *bFGF* and *HIF1a* in pancreatic cancer. However, in our patient group, the association between these two genes was significant, suggesting that the *bFGF*–*HIF1a* relationship also has a role in pancreatic cancer, especially because of the impact on prognosis both factors had. The survival probability was significantly higher with  $P = .013$  of patients with lower *bFGF* expression. As previously mentioned, the impact of *HIF1a* expression on prognosis was the strongest genetic factor on our patient cohort and even stronger than clinicopathologic parameters.

Although members of the PDGF family and their receptors have recently been discussed as potential drug targets in cancer [10], little is known about their role specifically in pancreatic cancer. As of today, especially the connection between *PDGFA* and *HIF1a*, although described in renal cell carcinoma, seems to be unevaluated in pancreatic cancer [25]. So far, the correlated mRNA expression of *PDGFA* and *VEGF* in cancer has only been described in a few studies [26] but not in pancreatic cancer tissue. In our patient samples, the correlation between *PDGFA* and *VEGF* was significant at a level of  $P = .02$ . The correlation between *PDGFA* and *HIF1a* was significant at  $P = .03$ , and the survival probability of patients with a high *PDGFA* expression was significantly lower as of patients with a low expression of the same gene ( $P = .003$ ).

When using gene expression as an approach to classify tumors, one can always question whether generally more aggressive tumors are metabolically more active or whether the expression of certain genes leads to a more aggressive tumor. Whereas this question cannot, of course, be definitively answered from a correlative study, the fact that these gene expressions did associate with various metrics of tumor aggressiveness strengthens the hypothesis of cause, not effect. Identifying genes that are associated with more aggressive tumors is useful to form a candidate oncogene pool that is available for further work to more definitively address the cause-or-effect question, such as *in vitro* experiments where the genes in question are transfected into cells. For example, insertion of a mutated *p53* into cells has been used to demonstrate that this gene directly causes many different effects, but it had to be identified first as being associated with more aggressive tumors.

## Conclusions

The significant impact of a high *PDGFA* expression on survival probability ( $P = .003$ ), the significantly higher survival probability of patients with a low *bFGF* expression ( $P = .01$ ), and the importance of a high *HIF1a* expression ( $P = .009$ ) in comparison to all clinicopathologic parameters suggest that these three genes may contribute to a better understanding of the prognosis of patients with pancreatic cancer and may even play a crucial role for the distribution of patients to multimodal therapeutic regimens. Larger studies including patients treated with actual chemotherapeutics seem to be warranted.

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