

# Vascular Endothelial Growth Factor, FLT-1, and FLK-1 Analysis in a Pancreatic Cancer Tissue Microarray

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**BACKGROUND.** Measures of vascular endothelial growth factor (VEGF) expression in pancreatic cancer typically have been qualitative or semiquantitative. The objective of this study was to use a series of algorithms called AQUA that quantitatively assesses protein expression on tissue microarrays (TMAs) to compare in situ expression of VEGF and its primary receptors, VEGF receptor 1 (FLT-1) and VEGF receptor 1 (FLK-1), on a pancreatic cancer TMA.

**METHODS.** TMAs were constructed by arraying 1.5-mm cores from 76 samples of pancreatic adenocarcinoma (1996-2002) that were obtained from the archives of the Yale Department of Pathology. The staining for AQUA was similar to standard immunohistochemistry and involved antigen retrieval and the application of primary antibodies, but with epifluorescence detection. Slides were counterstained with 4',6-diamidino-2-phenylindole for nuclear visualization and cytokeratin for membrane visualization. The primary antibodies used were VEGF, FLT-1, FLK-1, and cytokeratin.

**RESULTS.** Disease stage was highly prognostic for outcome, as expected. Total amounts of VEGF and its receptors were assessed within the tumor mask and were divided into quartiles. Kaplan-Meier survival curves showed that VEGF and FLK-1 were not associated clearly with outcome. However, the expression of FLT-1 was correlated significantly, and the patients who had tumors with the lowest expression FLT-1 levels had the worst survival ( $P = .0038$ ). In multivariate analysis, FLT-1 expression was an independent prognostic factor for overall survival ( $P = .0044$ ).

**CONCLUSIONS.** VEGF and its 2 principal receptors were expressed to varying degrees in tumors of the pancreas. A significant association was found between low expression of FLT-1 and both poor prognosis and advanced stage, suggesting that tumor expression of this VEGF receptor is a marker of less aggressive disease.

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**KEYWORDS:** angiogenesis, FLT-1, tissue microarray, quantitative image analysis.

Angiogenesis has been implicated in tumor growth and spread, because it was observed that tumor growth often was associated with increased vascularity and that transplanted tumors could induce a strong neovascular response. Subsequently, many molecules have been implicated in this process, including growth factors, cytokines, and angiopoietins. The vascular endothelial growth factor (VEGF) signaling pathway represents one of the most critical steps in this process.<sup>1</sup>

VEGF (also called VEGF-A) belongs to a family of related genes. Alternative splicing produces four principal isoforms: VEGF121, VEGF165, VEGF189, and VEGF206. VEGF121 does not have heparin-binding capacity (believed to endow mitogenic property to VEGF) and is freely diffusible, whereas the 189 and 206 isoforms are sequestered in the extracellular matrix; 165, which is the predominant isoform, is

intermediate.<sup>2</sup> VEGF has clear *in vitro* and *in vivo* activity promoting endothelial cell (EC) growth. In addition, it has been shown that VEGF has antiapoptotic effects, which are greater in newly formed peritumoral vasculature than in established vessels; chemotactic properties on bone marrow-derived hematopoietic cells; and induction of vascular permeability and vasodilatation.<sup>2</sup>

The receptors for VEGF initially were identified on ECs, because it was noted that VEGF had potent *in vitro* stimulatory effects on ECs,<sup>3</sup> but the receptors also were identified subsequently on bone marrow-derived hematopoietic cells.<sup>4</sup> The 2 primary receptors VEGFR-1 (FLT-1) and VEGFR-2 (FLK-1, KDR) are receptor tyrosine kinases. The precise function of FLT-1 in angiogenesis has not been established. Placenta growth factor (PlGF) activates FLK-1 by binding to FLT-1 and transphosphorylating FLK-1, which has a proangiogenic effect, yet ligand-mediated FLT-1 autophosphorylation is weaker than FLK-1 autophosphorylation, and it is known that a juxtamembrane repressor motif of FLT-1 impairs phosphoinositide-3 kinase signaling and EC migration.<sup>2</sup> Indeed, an alternatively spliced, soluble form of FLT-1 that lacks a portion of the extracellular domain and the transmembrane and intracellular domains also has been identified in both physiologic and pathologic conditions. It has been hypothesized that both this form and the full-length, membrane-bound form play a decoy role by modulating endogenous VEGF activity through diminished binding to FLK-1.<sup>5</sup> Conversely, FLK-1 is considered the major mediator of VEGF's proliferative, angiogenic, and permeability-enhancing effects. Key evidence is the failure of FLK-1 null mice to develop blood islands or any organized blood vessels, resulting in embryonic lethality.<sup>6</sup>

The results from *in situ* hybridization studies that demonstrated VEGF mRNA up-regulation in many human cancers suggested more directly a role for VEGF in carcinogenesis.<sup>7</sup> Although most studies have localized VEGF expression to tumors and its receptors FLT-1 and FLK-1 to ECs, suggesting a paracrine angiogenic effect of VEGF, other investigators have demonstrated more ubiquitous expression, with VEGF receptors also present in tumor cells.<sup>8,9</sup> This has led to hypotheses that autocrine VEGF/receptor loops support tumor growth and survival through nonangiogenic, growth-promoting mechanisms. Furthermore, although it has been shown that VEGF and its receptors are expressed in pancreatic cancer, the significance of this is uncertain; because some (but not all) studies have shown a correlation between VEGF expression, vessel density, and advanced disease.<sup>10-13</sup> Nevertheless, a number of antiangiogenic agents have

been developed for clinical trials, including neutralizing antibodies to VEGF or VEGF receptors, soluble VEGF/VEGFR hybrids, multitargeted tyrosine kinase inhibitors, and direct EC toxins.<sup>14</sup>

To further characterize the expression and clinical significance of VEGF, FLT-1, and FLK-1, we analyzed the expression of these markers on an extensively annotated pancreatic adenocarcinoma tissue microarray (TMA) with a modified immunohistochemistry technique. These slides were analyzed with our recently developed series of algorithms called AQUA, which can assess TMAs by using fluorescent tags to define tumors, localize subcellular compartments, and grade the intensity of specific markers on a continuous scale.<sup>15</sup> This technology reduces the bias of subjective assessment and allows quantitative assessment of protein expression by using molecular colocalization techniques.

## **MATERIALS AND METHODS**

### **Patient Characteristics**

Our cohort consisted of 76 patients with pancreatic ductal adenocarcinomas from whom tissue was acquired between 1996 and 2002. Extensive demographic, pathologic, treatment, and survival information was derived from review of patient charts, pathology reports, and the Yale and Connecticut Tumor Registries. The clinical information and tumor blocks were retrieved with approval of the Yale University Human Investigation Committee. Because a substantial proportion of patients had advanced-stage disease at presentation, available tissues from these patients sometimes were from nonpancreatic sources, such as liver. Only surgical resections or biopsies were included, and any patients with only cytopathologic specimens were excluded. Patient characteristics are summarized in Table 1.

### **Tissue Microarray**

Formalin fixed, paraffin embedded blocks from identified patients were retrieved from the archives of the Yale University Department of Pathology with approval of the Yale University Human Investigation Committee. Representative areas of invasive tumor were identified, and 1.5-mm-diameter cores were placed into a recipient block by using a precision arraying instrument (Beecher Instruments, Silver Spring, MD). Five-micrometer sections were affixed to adhesive slides using an ultraviolet, cross-linkable, tape transfer system (Instrumedics Inc., Hackensack, NJ), then coated in paraffin, and stored in a nitrogen chamber prior to staining to prevent antigen oxidation and degeneration.<sup>16</sup>

**TABLE 1**  
**Patient Cohort Characteristics**

Characteristic	No. of Patients (%)
Median age (range), y	61 (44-83)
Male	34 (45)
Disease stage*	
Stage I	4 (5)
Stage II	32 (43)
Stage III	11 (14)
Stage IV	29 (38)
Treatment	
Primary surgery	30 (39)
Chemotherapy	36 (47)
Radiation	19 (25)
Tissue source	
Pancreas	38 (51)
Liver	16 (21)
Other	21 (27)
Not recorded	1 (1)
Tissue source (AQUA evaluable for FLT-1 expression)	
Pancreas	33 (64)
Liver	9 (18)
Other	9 (18)
Median follow-up (range), m	9 (0.1-82.0)

FLT-1: vascular endothelial growth factor receptor 1.

\* Stage was determined according to American Joint Committee on Cancer criteria.

### Immunohistochemistry

Staining procedures for TMA slides with AQUA have been described previously.<sup>15</sup> Briefly, slides were deparaffinized in xylene, rinsed in ethanol, and rehydrated. Antigen retrieval was performed by pressure cooking for 15 minutes in 6.5 mM sodium citrate buffer. Endogenous peroxidase was quenched by immersing the array in a 2.5% methanol/hydrogen peroxide buffer for 30 minutes. Nonspecific background staining was minimized further by preincubating the array with 0.3% bovine serum albumin in 0.1 M Tris-buffered saline, pH 8.0, for 1 hour. The antibodies used were anti-VEGF, a polyclonal antibody against the amino terminus; anti-FLK-1, a monoclonal antibody against the carboxy terminus; and anti-FLT-1, a polyclonal antibody also against the carboxy terminus<sup>11</sup> (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Because anti-FLT-1 antibody is against the intracellular domain of FLT-1, it presumably does not recognize the soluble form of FLT-1. Anti-VEGF recognizes the 121, 165, and 189 isoforms and has been used in numerous prior studies.<sup>10,12,13,17,19</sup> For the purposes of our automated analysis, tumor cells also were differentiated from stroma with an anticytokeratin antibody (DAKO, Carpinteria, CA). This primary antibody cocktail was incubated overnight at 4°C in a humidified chamber. Goat antimouse or rabbit anti-body conjugated to a horseradish peroxidase-deco-

rated dextran polymer backbone (Envision; DAKO Corp.) was used as a secondary reagent to detect the bound primary antibody, and indodicarbocyanine-tyramide was used to visualize the amplified signal. The cytokeratin was visualized with an indodicarbocyanine-conjugated secondary antibody; then, the array was counterstained with 4',6-diamidino-2-phenylindole (DAPI) to localize nuclei.

### Automated Image Acquisition and Analysis

Image acquisition and automated analysis also have been described extensively in our previous work.<sup>15</sup> Briefly, slides are scanned using a customized, computer-controlled epifluorescence microscope (Olympus BX-51 with xy-stage and z controller). Images of each spot at different wavelengths are acquired automatically with a high-resolution monochromatic camera (CookePCO). Because the AQUA software initially was developed for TMA spots of 0.6 mm diameters instead of our 1.5-mm spots, 4x images of each corner of each spot were acquired in an automated fashion, and these were then "stitched" together to compose the entire spot. Then, with this stack of uncompressed images, the AQUA software can distinguish between areas of tumor and stromal elements by using the cytokeratin stain, resulting in a unique binary cytokeratin tumor mask for each spot. Furthermore, the cytokeratin and DAPI stains are used to assign each pixel under the tumor mask into nonoverlapping membrane, cytoplasmic, and nuclear locales. AQUA scores for VEGF and its receptors are then calculated that correspond to the average signal intensity divided by locale area. This information can be exported into a format that is suitable for analysis by standard software packages.

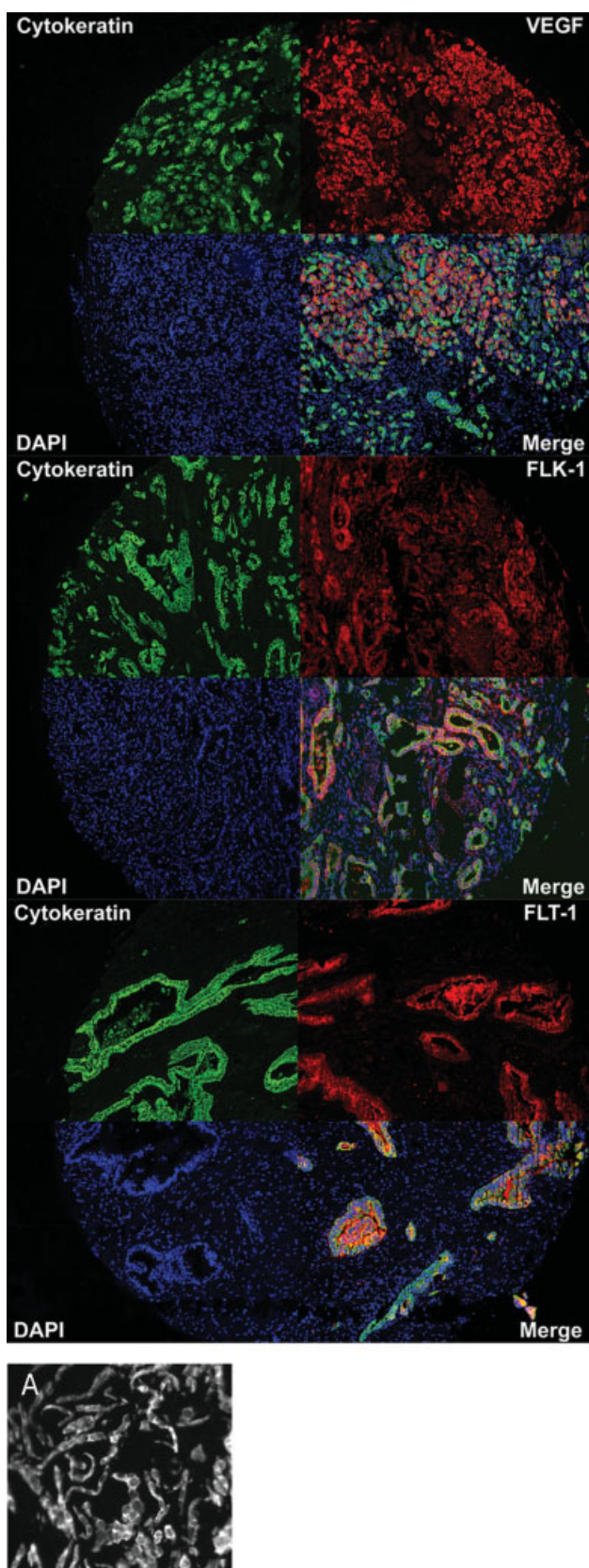
### Statistical Analysis

All analyses were performed using Statview software (version 5.0.1; SAS Institute Inc., Cary, NC). The Cox model was used to perform univariate analyses to determine the relative risk of each variable. Correlations between markers and clinicopathologic parameters were determined using chi-square analysis. Survival curves were calculated using Kaplan-Meier analysis with assessment of statistical significance using the Mantel-Cox log-rank test. All work in this study was approved by the Yale University Human Investigations Committee.

## RESULTS

### Immunostaining Patterns

VEGF staining showed a predominantly membrane/cytoplasmic (i.e., nonnuclear) distribution in tumors (Fig. 1). Nuclear staining was significantly less. FLT-1



also was predominantly nonnuclear with relatively little localized to the nucleus. Conversely, FLK-1 stained fairly diffusely within the tumor. Most immunostaining reports of FLK-1 show a predominant cytoplasmic locale, although FLK-1 nuclear translocation by VEGF binding has been reported in cultured ECs.<sup>20</sup> When subsequent outcome associations were determined for the predominant compartments for the 3 biomarkers (i.e., nonnuclear for VEGF and FLT-1 and nonnuclear and nuclear for FLK-1), there were no significant differences compared with the results obtained simply from the tumor mask. Thus, to assess total staining in the best manner, all of our subsequent analyses used AQUA scores within the tumor mask. Although subcellular localization may be important, the current limits of resolution of the system prevented more detailed analysis of this issue. Primary antibodies also were used on cell line microarrays. For example, T47D showed strong membranous expression of FLT-1 (see Fig. 1).

#### Validation of Microarray Cohort

We used 1.5-mm diameter cores for TMA histospots rather than the more standard 0.6 mm because of the characteristically abundant desmoplastic tissue surrounding pancreatic adenocarcinomas. It was determined that samples were nonevaluable if there were technical problems or a lack of malignant tissue. The mean number of evaluable samples per marker was 48 (63%) in this 76-sample array. Although 48% of samples on the TMA were from nonpancreatic tissue sources, the evaluable cohort consisted of only 36%, mainly because the amount of tumor cells present from metastatic sites was less than the amount present from the primary pancreas site (Table 1).

To validate our cohort of patients, we assessed several established indicators of prognosis in pancreatic cancer. Univariate analysis showed that disease stage and resection of primary tumor (probably a reflection of stage) were associated significantly with 5-year overall survival (Table 2). In our cohort, the lowest level of FLT-1 expression also was associated significantly with poor overall survival when it was analyzed as a continuous variable or as a binary vari-

**FIGURE 1.** Immunostains for vascular endothelial growth factor (VEGF) and its receptors, VEGFR-1 (FLT-1) and VEGFR-2 (FLK-1). Representative high expresser histospots of the pancreatic tissue microarray stained with VEGF, FLT-1, and FLK-1. The 4 panels per marker are stitched together images of the four quadrants of each spot. *Inset A* shows a cell line microarray of T47D that had strong membrane expression of FLT-1. DAPI: 4',6-diamidino-2-phenylindole.

**TABLE 2**  
**Univariate and Multivariate Analyses of 5-Year Overall Survival:**  
**Cox Regression**

Marker	P	HR (95% CI)
Univariate analysis		
Age (continuous variable)	.9055	
Female	.447	0.824 (0.501-1.357)
Stage IV disease	.0023*	2.37 (1.36-4.13)
Grade < 2	.1602	1.702 (0.810-3.576)
Primary tumor resected	.0019*	0.41 (0.23-0.72)
Negative margins (patients who underwent primary resection)	.0907	0.43 (0.160-1.144)
AQUA tumor mask		
VEGF (bottom vs. top quartiles) <sup>†</sup>	.7202	1.186 (0.466-3.021)
FLK-1 (bottom vs. top quartiles) <sup>†</sup>	.2212	2.062 (0.647-6.572)
FLT-1 (continuous variable)	.0357*	
FLT-1 (bottom vs. top quartiles) <sup>†</sup>	.0062*	4.43 (1.526-12.857)
Multivariate analysis		
Female	.8175	0.905 (0.387-2.116)
Stage IV disease	.5389	0.678 (0.196-2.342)
Grade < 2	.6862	1.199 (0.498-2.886)
FLT-1 AQUA tumor mask quartiles	.0044*	9.872 (2.042-47.738)

HR: hazard ratio; 95% CI: 95% confidence interval; VEGF: vascular endothelial growth factor; TM: tumor mask; FLK-1: VEGF receptor 2; FLT-1: VEGF receptor 1.

\* Significant at  $P < .05$ .

<sup>†</sup> Bottom versus top 25% of expression, i.e., the relative risk for the bottom 25% compared with the relative risk for the top 25%.

able using the bottom quartile compared with the top quartile.

### Correlation of VEGF, FLT-1, and FLK-1 Expression and Outcome

Mean AQUA scores for VEGF, FLT-1, and FLK-1 within the tumor masks were 10.0, 16.8, and 26.5. When these scores were divided into traditional quartiles, Kaplan-Meier survival curves did not show a clear trend for either VEGF or FLK-1 with respect to levels of expression and survival (Fig. 2). Although VEGF did show significance when the  $P$  value cut off was set at  $< .05$ , it was not a linear correlation, and the second quartile had the greatest degree of separation. Nevertheless, because a U-shaped distribution of expression in relation to outcome is possible, the relatively small numbers of patients in each quartile make such definitive conclusions impossible. However, FLT-1 was associated significantly with survival, with a median survival of 5 months for the lowest quartile compared with 19 months for the highest quartile. Multivariate analysis also showed that FLT-1 remained a significant prognostic variable for overall survival independent of gender, stage, and grade (Table 2).

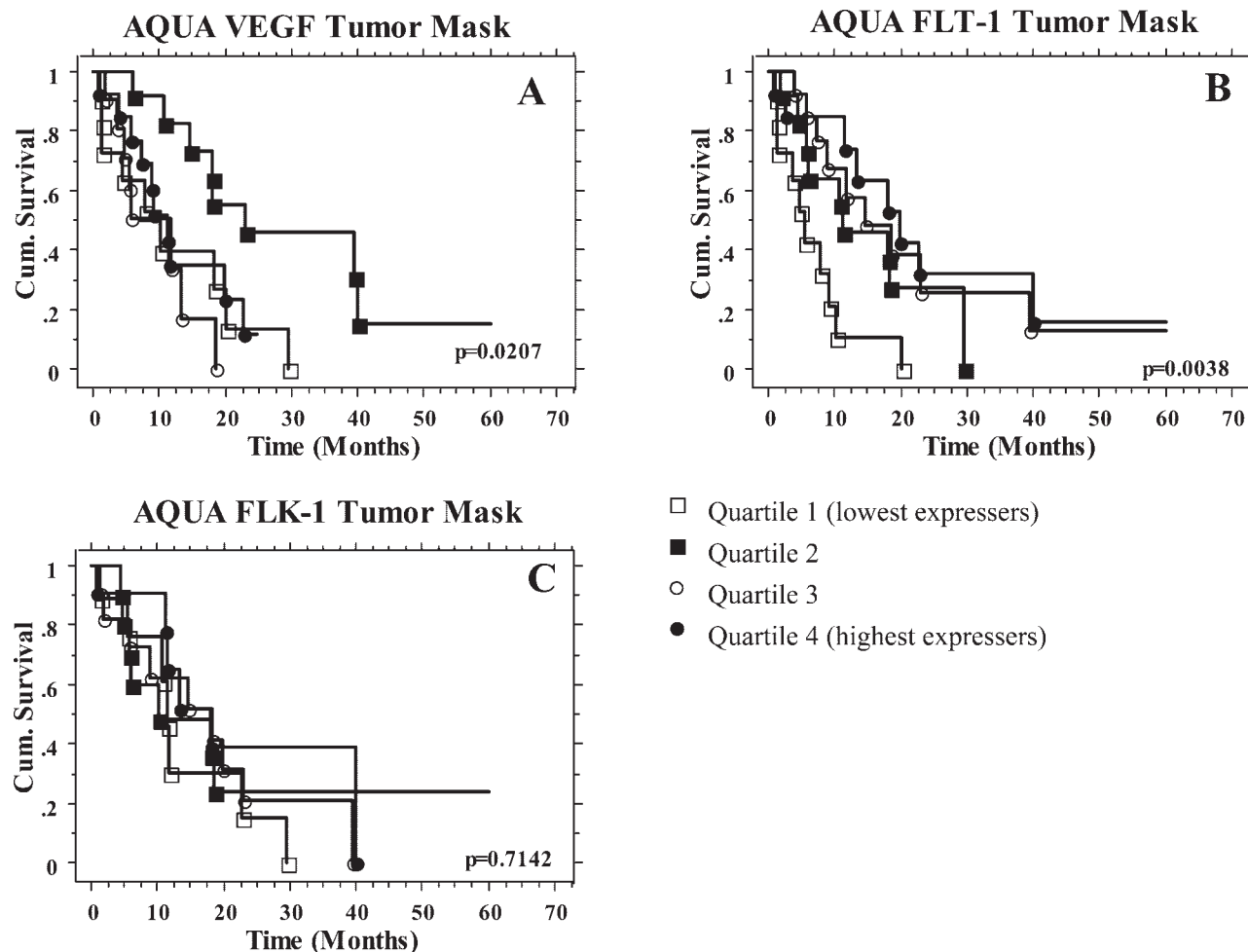
### Association with Other Biomarkers

We also performed a chi-square analysis to determine possible associations between the different markers (Table 3). This analysis showed that high FLT-1 expression was associated significantly with high levels of VEGF and FLK-1 but that VEGF and FLK-1 were not associated significantly with each other. FLT-1, however, was the only marker that was highly correlated with disease stage (i.e., low FLT-1 expression was associated with advanced stage). In addition, nonpancreatic tissue sources were associated with high stage (most of these were liver biopsies from metastatic liver sites) and with low FLT-1 expression.

### DISCUSSION

We used our automated imaging system with molecular colocalization techniques to assess the prognostic significance of VEGF and its 2 principal receptors on our pancreatic cancer TMA. Immunostaining patterns showed a predominant membrane/cytoplasmic locale for VEGF and FLT-1 but a more diffuse, subcellular expression pattern for FLK-1 with higher nuclear scores. Whereas VEGF and FLK-1 were not associated significantly with survival, low FLT-1 expression was associated with worse overall survival and with advanced stage of tumor.

One difficulty with our study was the construction of TMAs from patients with small-core biopsies and surgical resections. Redundant cores, therefore, were not feasible for many patients, especially because larger 1.5-mm cores were used for our array. However, many studies now report adequate representation of a variety of tumors with  $\leq 2$  cores.<sup>21,22</sup> Several recent studies have used TMAs for pancreatic cancer with larger diameter cores, in the range of 0.6 mm to 2.0 mm.<sup>23-25</sup> Swierczynski et al. showed that, although a 4-fold redundant pancreatic adenocarcinoma TMA with 1.4-mm cores showed exceptional results with only a 1% failure rate secondary to the lack of malignant tissue and a 5% failure rate secondary to technical problems,<sup>26</sup> failure rates in many malignancies typically are much higher.<sup>27</sup> We had a 37% overall failure rate secondary to a lack of tissue cores, a lack of malignant tissue, or technical reasons. In the current study, as noted above, 48% of arrayed samples were from nonpancreatic tissue sources, which may have more intrinsic variability, and consecutive slides were stained with hematoxylin and assessed for adequate tumor representation, and no tumor/minimal tumor spots were excluded from the analysis. Staining for automated analysis requires additional steps and may damage tissue to a greater extent; and, during AQUA analysis, rigorous cut-off levels are used, such that any



**FIGURE 2.** Kaplan–Meier survival curves of vascular endothelial growth factor (VEGF; A) and its receptors, VEGFR-1 (FLT-1; B) and VEGFR-2 (FLK-1; C) quartiles and overall survival. VEGF expression in tumor mask did not show a clear association with survival. Rather, the second quartile appeared to be associated with better survival. FLT-1 survival curves were nearly overlapping, and FLT-1 showed a clear trend with the lowest expressers doing worst.

**TABLE 3**  
Chi-Square Analysis P Values

Marker	Stage IV	Biopsy Site	P	
			AQUA TM quartiles	
			VEGF	FLT-1
Stage IV				
Biopsy site	< .0001*†			
VEGF AQUA TM quartiles	.2969	.1887		
FLT-1 AQUA TM quartiles	.0006*‡	.0008*‡	.0021*	
FLK-1 AQUA TM quartiles	.7018	.8928	.3598	.0178*

VEGF: vascular endothelial growth factor; TM: tumor mask; FLT-1: VEGF receptor 1; FLK-1: VEGF receptor 2.

\*  $P < .05$ .

† Pancreatic tissue source associated with low stage and with high FLT-1.

‡ Inverse correlation.

spot below a predetermined threshold level for detectable fluorescence is excluded. Table 1 shows that a greater proportion of nonpancreatic tissue specimens, compared with pancreatic specimens, were excluded from the final analysis, probably because of the reasons described above. Thus, our analysis was biased for patients with primary pancreatic specimens available.

Although it is believed that VEGF promotes angiogenesis by stimulating its receptors on tumor-associated ECs, its role in tumor growth and spread probably is much more complex. Studies also have suggested a possible autocrine effect for VEGF and VEGF receptors by demonstrating the presence of both the ligand and the receptors in tumor cells.<sup>8,9</sup> This also has been demonstrated in pancreatic cancer samples and in cell lines.<sup>11,17</sup> Although some have shown functional autocrine VEGF/VEGFR loops that support in vivo tumor

growth and survival, the precise mechanisms behind this potential "nonangiogenic" and non-EC-related pathway for VEGF is unclear.<sup>28</sup>

In pancreatic cancer, several studies have examined the functional and prognostic significance of VEGF and VEGFR expression. Most of those studies used immunohistochemistry to show that VEGF was expressed in the majority of cancers and that expression in general was correlated with a worse outcome and markers of poor prognosis, such as advanced stage.<sup>12,13,18,29</sup> For example, Itakura et al. demonstrated VEGF mRNA and protein expression in all pancreatic cell lines tested and used immunohistochemistry to demonstrate a predominant membrane/cytoplasm localization of VEGF that was associated with vessel density, tumor size, and local spread, but not with survival.<sup>13</sup> Their staining results were confirmed with in situ hybridization studies. However, other studies have produced conflicting results with no correlations with outcome.<sup>10,19</sup> Follow-up studies from Itakura et al. also confirmed the coexpression of VEGF and FLT-1 and FLK-1 in cultured pancreatic cancer cell lines and in tumor cells on tissue specimens, and serial sections showed that all 3 often were colocalized in cancer cells.<sup>17</sup> Buchler et al. demonstrated similar findings<sup>11</sup>: It is noteworthy that their results study showed that FLK-1 (but not FLT-1) was correlated with shorter survival and that antisense oligonucleotides for FLK-1 (but not FLT-1) abrogated the in vitro stimulatory effects of VEGF on pancreatic cancer cell proliferation, suggesting that the growth-promoting effect of VEGF in pancreatic cancer may be dependent predominantly on FLK-1 rather than FLT-1.

FLT-1 has shown a divergent effect, both negative and positive, in tumor growth potential. The conflicting literature may reflect the fact that it likely has different functions, depending on the physiologic and pathologic conditions and tissue type in which it is expressed. In the current study, low tumor FLT-1 expression was associated with worse survival and was correlated with advanced disease stage. This suggests that it may have a protective effect and act as a negative regulator of angiogenesis. Although our FLT-1 antibody presumably only detected full-length FLT-1 and not the soluble form, because it recognizes the intracellular domain, potential decoy functions for this receptor have been attributed to both forms.<sup>30</sup> Furthermore, studies in ECs have shown high affinity but weaker tyrosine autophosphorylation and signaling in response to VEGF when binding to FLT-1 compared with FLK-1.<sup>31</sup> In addition, potentiation of VEGF-mediated mitogenesis by other growth factors, such as PlGF, through possible displacement of VEGF from

FLT-1 binding onto FLK-1 has been postulated.<sup>30</sup> The specific downstream effects of the VEGF/FLT-1 interaction in pancreatic tumor cells have not been studied, and it is possible that a similar decoy effect may be functioning for FLT-1 in pancreatic cancer. Finally, although this correlation has not been reported previously in pancreatic cancer, studies in breast cancer have shown an association of low FLT-1 expression with a worse prognosis<sup>32</sup> and an association of high intratumoral soluble FLT-1 and VEGF ratios with favorable outcomes.<sup>33</sup> It is interesting to note that we only analyzed tumor expression and not the stromal or EC localization of FLT-1. Future studies should characterize the expression of soluble FLT-1 and full-length FLT-1 expression further in the various compartments and should analyze the intracellular effects of ligand binding to FLT-1 in pancreatic cancer.

In conclusion, using TMA and the AQUA technologies as a platform, we showed that VEGF, FLT-1, and FLK-1 were expressed variably in pancreatic adenocarcinomas and that FLT-1 was correlated significantly with disease stage and patient survival. These findings support a role for this pathway in the natural history of pancreatic cancer and, in the future, may have implications in the design of studies of antiangiogenic therapies in pancreatic cancer.

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