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Tumor angiogenesis as a prognostic predictor in pancreatic cancer

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Abstract The purpose of this study was to evaluate the role of angiogenesis, proliferative activity (assessed by Ki-67 expression), *p53* and *ras*-oncogene (H-*ras*) expression, and conventional clinicopathologic factors in predicting overall survival rates in patients with pancreatic ductal adenocarcinoma. We followed-up 22 patients with ductal adenocarcinoma of the pancreas for a median of 19 months (range, 2 to 44 months). Angiogenesis was quantitated as vascular surface density (VSD) and the number of vessels per mm^2 stroma (NVES) after microvessels were immunostained, using factor VIII-related antigen. p53, H-ras, and Ki-67 proteins were also determined immunohistochemically. VSD and NVES showed significant correlations with increased proliferative activity, poor tumor differentiation, and tumor size of 3 cm or more ($P = 0.001$, $P = 0.013$, and $P = 0.047$, respectively). The overall 2-year survival rate of 33.3% in patients with high VSD and NVES values was significantly worse than that of 66.6% estimated in patients with low microvessel count (log rank, 3.97; $P = 0.046$). In multivariate analysis using the Cox model, VSD was found to be an independent prognostic factor of survival ($P = 0.039$). H-*ras* and *p53* expressions were not correlated with angiogenesis parameters. We conclude that, in pancreatic ductal adenocarcinoma, angiogenesis is closely related to tumor growth and patient survival.

Key words Pancreatic cancer · Surgery · Angiogenesis · Survival

Introduction

Pancreatic cancer remains a highly lethal malignancy. Although significant advances have been made in improving the safety of pancreatic cancer resection, only modest improvements have occurred in overall 5-year survival rates.[1] A number of clinicopathological prognostic factors (e.g., age, sex, tumor size and grade, lymph node involvement, and perineural and retroperitoneal invasion) have been considered to predict the clinical outcome in pancreatic cancer.[2,3] However, recently, molecular and immunohistochemical approaches have been used to elucidate the inherited biological aggressiveness of pancreatic adenocarcinoma and to suggest avenues for the development of novel treatment strategies. Considerable importance has been attributed to the role of *ras*-gene mutations and the inactivation of tumor suppressor genes in pancreatic carcinogenesis.[1,4,5] In particular, the *p53* gene plays a key role in cell cycle regulation, implying that alterations of the *p53* gene may be associated with increased proliferative activity and unlimited cell growth.[6] Experimental evidence has also shown that, after reaching a size of about 1–2 mm^3, tumor growth and spread, and metastases, were strictly dependent on angiogenesis.[7–9] This so-called neovascularization may be stimulated by factors secreted by tumor cells or tumor-associated inflammatory cells, or by the extracellular matrix.[9,10] Recent data show that angiogenesis has been detected as early as at the transition from hyperplasia to neoplasia.[1,4,11–13] In addition, tumor angiogenesis, quantitated by microvessel counting, has been correlated with a less favorable prognosis in a number of human cancers, including breast cancer,[14] cutaneous malignant melanoma,[15] prostate carcinoma,[16] lung cancer,[17] and colorectal cancer.[18,19] The extent of neovascularization is also an independent prognostic factor in brain tumors[20] and in invasive breast carcinoma.[21] However, the prognostic significance of angiogenesis and the role of major molecular vehicles such as *p53* and *ras*-oncogenes in pancreatic tumorigenesis have not been investigated extensively thus far.

The aims of this investigation were twofold: (a) to determine whether the quantitation of angiogenesis, *p53* and H-*ras* mutations, and proliferative activity can be documented in pancreatic ductal adenocarcinoma, and (b) to assess whether these determinants can be

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correlated to tumor aggressiveness and provide prognostic information for survival.

Patients and methods

Patients

Twenty-two patients with potentially curable pancreatic cancer without peritoneal seeding, extraperitoneal organ metastases, and superior mesenteric artery involvement, and with a confirmed histological diagnosis of ductal adenocarcinoma of the pancreas, were included in the study. All patients underwent standard pancreaticoduodenectomy with curative intent. The sex ratio was 11:11 and the median age was 62 years (range, 41 to 82 years). The extent of pancreatic tumor invasion/metastasis was based on the TNM staging system proposed by the American Joint Committee on Cancer.[22] Ten (45.5%) patients were classified with stage I, one (4.5%) with stage II, and 11 (50%) with stage III disease.

The following clinicopathological data were analysed: (a) tumor characteristics, including tumor size and grade, lymph node and perineural involvement, and proliferative activity; (b) treatment variables, including surgical margins and retroperitoneal invasion; and (c) final outcome. Tumor diameter is the maximum diameter of tumor obtained from the pathologic gross description. A three-tiered grading system (well, moderately, and poorly differentiated) was used to classify histologic differentiation.[23]

Immunohistochemical staining

The tumor tissues obtained from archival paraffin blocks were originally fixed in formalin. The blocks were sectioned onto poly-L-lysine-coated slides. The avidin-biotin-peroxidase method was employed, using primary monoclonal antibodies against *p53* (prediluted; Dakocorp, Glostrup, Denmark), H-*ras* (1:40; Dakocorp), Ki-67 (prediluted, Dakocorp), and factor-VIII-related antigen (1:100; Dakocorp). Briefly, the sections were deparaffinized, and endogenous peroxidase activity was blocked by using a 0.3% solution of hydrogen peroxidase in phosphate-buffer solution (PBS) at room temperature for 10 min. After trypsin treatment for anti-factor-VIII-related antigen antibody, and microwave treatment for anti-*p53* and anti-Ki-67 antibodies, primary antibodies were applied for 30 min at room temperature, and the sections were washed in PBS. Linking antibody and streptavidin-peroxidase complex (Dakocorp) were added consecutively for 10 min at room temperature and the sections were washed in PBS. The peroxidase activity was visualized with diaminobenzidine (Sigma, St. Louis, MO, USA) applied for 5 min. Appropriate tissue sections as positive controls for each primary antibody were also labeled.

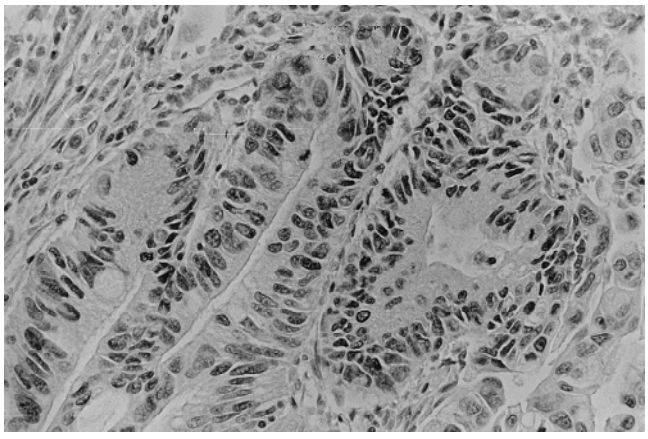

Fig. 1. Strong nuclear *p53* immunopositivity of pancreatic tumor cells. Immunoperoxidase, ×200

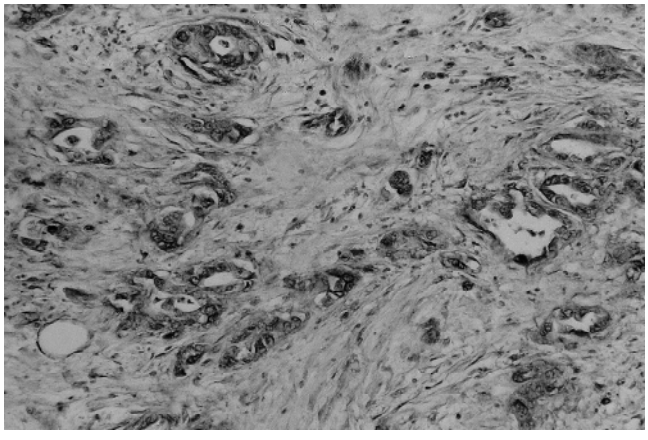

Fig. 2. Cytoplasmic H-*ras* immunopositivity of an infiltrating pancreatic ductal carcinoma. Immunoperoxidase, ×100

The most representative areas of the section, including the tumor tissue with the poorest histologic differentiation, were selected and marked for the analysis. The immunostaining for *p53* and H-*ras* proteins was evaluated as either positive or negative (Figs. 1 and 2). The degree of positive staining for Ki-67 protein was evaluated by scoring on a scale for 1 to 4 for intensity (I) (i.e., none, mild, moderate, and strong), and for distribution (D) (i.e., none, focal, patchy, and diffuse).[24] Tissues with I × D less than or equal to 4 were considered weakly positive, and those with I × D greater than 4 were designated strongly positive (Fig. 3).

Stereologic measurements

Vascular structures were labeled immunohistochemically, using factor VIII- related antigen. The most repre-

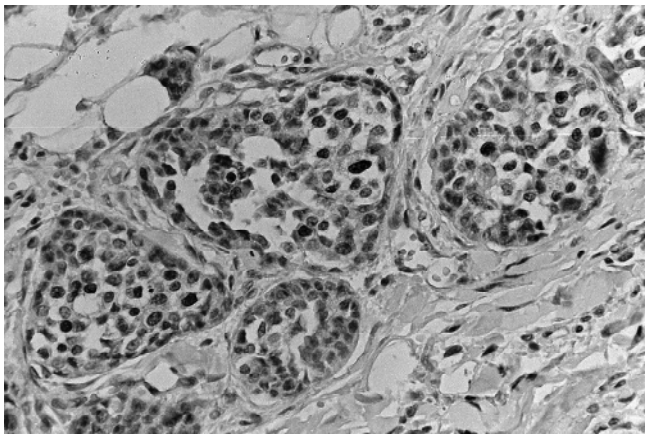

Fig. 3. Strong nuclear Ki-67 immunopositivity of pancreatic ductal carcinoma. Immunoperoxidase, ×200

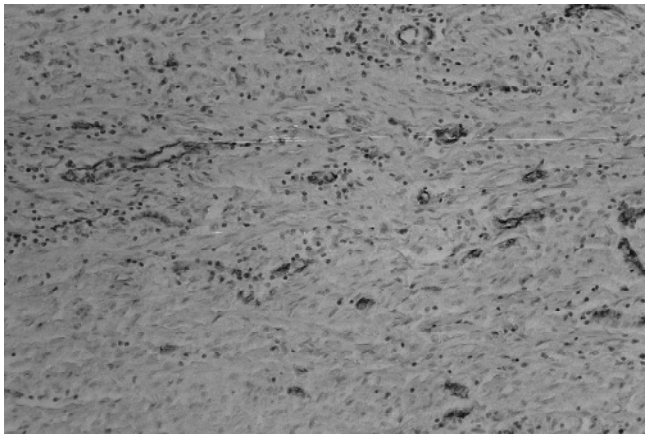

Fig. 4. Immunolabeling of vascular structures, using factor-VIII-related antigen. Immunoperoxidase, ×100

sentative areas of the section, including three hot spots in the periphery of the tumor with the highest angiogenetic activity, were selected and marked for the analysis (which was performed by two pathologists [E.Ö. and Ö.S.] without either knowing each other's selection and patients' clinical outcome) (Fig. 4). The microscopic image obtained at a ×10 objective was projected by a change-coupled device (CCD) camera (Sony, Tokyo, Japan) to a monitor (Sony) attached to a microscope (Nikon, Tokyo, Japan). The final magnification of the microscopic image including the selected area was ×525. The representative area of the image was superimposed with a transparent grid containing 11 horizontal and 11 vertical test lines with known total test line length (Lr; 10.34 μm). The vascular surface density (VSD), equivalent to the vascular surface area (mm^2) per unit tissue volume (mm^3), and the number of vessels per mm^2 stroma (NVES) were computed according to the following formulas:[25]

$$VSD = \frac{\sum \ln \cdot 2 \cdot 121}{Istr \cdot Lr} \qquad NVES = \frac{N \cdot 121}{Istr}$$

where Istr = the number of the grid points superimposed on the stroma, In = the counts of intersections between test lines and vessel walls, and N = the number of vessels within the measuring field. The coefficient error of the stereologic method of measuring VSD and NVES was kept equal to or less than 5%.

Statistical analysis

Statistical analysis was performed on a personal computer with SPSS statistical software (SPSS, Chicago, IL, USA). The probability level of 0.05 or less was chosen to represent statistical significance. Group means were compared using Student's *t*-test. In addition to Spearman's correlation test, Fisher's exact test was used to calculate *P* values for immunohistochemical evaluation, as the cell frequencies were too small for the standard χ^2 test to be accurate. Survival curves were calculated using the Kaplan-Meier method and analyzed by the log-rank test. To assess the influence of variables on survival, multivariate analysis was performed by the Cox proportional hazard regression model.

Results

Patients

Fourteen patients died between 2 and 37 months after the operation (median, 14 months). The remaining 8 patients were alive with a median follow-up period of 23.5 months (range, 19 to 44 months). The overall median survival of these 22 patients was 19 months (range, 2 to 44 months). The 1-year survival rates were 81.8% for those with stage I and 63.6% for those with stage III disease. The 2-year survival rates were 63.6% and 30.3% for stage I and III, respectively (Fig. 5).

Immunohistochemical findings and clinicopathological correlations

There was no significant correlation between angiogenesis and clinicopathologic factors such as sex, depth of invasion, lymph node metastasis, stage, or retroperitoneal and perineural invasion. VSD and NVES showed significant correlations with increased proliferative activity, poor histologic differentiation, and tumor size of 3 cm or more. VSD and NVES were also analyzed in relation to other variables, including H-*ras* and *p53* immunostaining. However, VSD and NVES were not correlated with any of these parameters (Table 1).

Considerable intratumor heterogeneity was observed in the distribution of stained microvessels, and the mean VSD and NVES values of hot spots at the invasive tumor margin were 3.43 ± 1.07 and 21 ± 5.61, respectively. Patients were stratified into two subgroups on either side of the mean value of VSD and NVES. The 2-year survival rate of 33.3% in patients with high VSD and NVES values was significantly worse than that of 66.6% estimated in patients with low microvessel count (log-rank, 3.97; $P = 0.046$) (Fig. 6). The effect of variables (sex; age; tumor size; grade; perineural and retroperitoneal invasion; lymph node metastasis; *p53*, Ki-67, and H-*ras* expressions; VSD; and NVES) presumably associated with prognosis was studied by multivariate analysis using the Cox model. VSD proved to be an independent prognostic factor of survival (hazard ratio, 1.88; $P = 0.039$) (Table 2).

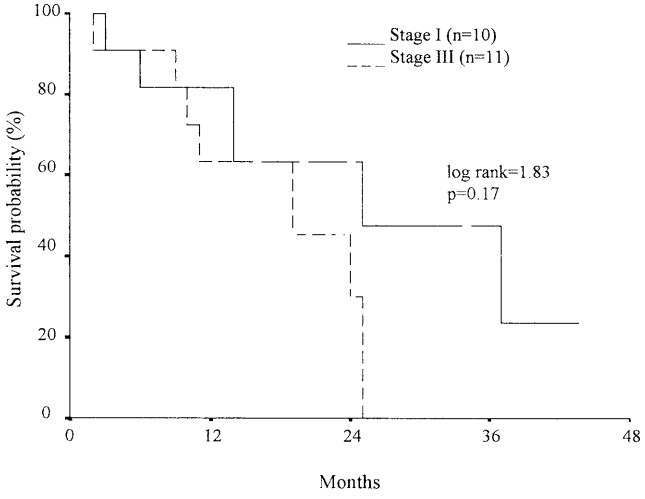

Fig. 5. Overall survival of patients with stage I ($n = 10$) and stage III ($n = 11$) pancreatic cancer

Of the 22 tumor tissues that were analyzed, *p53* immunopositivity was determined in 10 (45.4%) and was found to be significantly higher in tumors of 3 cm or more ($P = 0.03$). Also, 3 tumor tissues (13.6%) showed positive H-*ras* immunostaining. These were all stage I (T_2N_0) tumors and were negative for *p53* immunostaining. H-*ras* and *p53* expressions did not correlate with the parameters of angiogenesis.

Strong Ki-67 immunopositivity was found in 15 tumor tissues (68%), whereas 7 tumor tissues (32%) were weakly positive. On statistical analysis, Ki-67 expression

Table 1. Correlation between cliniopathologic variables and parameters of angiogenesis (VSD and NVES)

Variable	Number of patients	VSD (mm^{-1}) (mean ± SD)	*P*	NVES (mm^{-2}) (mean ± SD)	*P*
Total	22	3.4 ± 1.0		21 ± 5.6	
Sex					
Male	11	3.5 ± 1.1	0.8	20.9 ± 5.3	0.9
Female	11	3.4 ± 1.0		21.1 ± 6.2	
Histologic grade					
Well/moderate	14	3.0 ± 1.0	0.013	19.0 ± 5.6	0.025
Poor	8	4.2 ± 0.9		24.5 ± 3.7	
Depth of invasion					
T1–2	16	3.2 ± 1.0	0.052	19.7 ± 5.7	0.08
T3	6	4.2 ± 0.8		24.4 ± 3.6	
Tumor size					
<3 cm	8	2.8 ± 0.9	0.047	17.2 ± 5.4	0.042
≥3 cm	14	3.8 ± 1.1		24.6 ± 5.2	
Lymph node metastasis					
Negative	11	3.0 ± 0.8	0.09	19.3 ± 5.0	0.18
Positive	11	3.8 ± 1.1		22.6 ± 5.9	
Ki-67					
Weak	7	2.0 ± 0.7	0.001	15.8 ± 4.5	0.001
Strong	15	3.9 ± 0.9		23.4 ± 4.4	
p53					
Negative	12	3.3 ± 0.9	0.45	20.8 ± 5.1	0.85
Positive	10	3.6 ± 1.3		21.2 ± 6.5	
H-*ras*					
Negative	19	3.5 ± 1.2	0.81	20.9 ± 5.9	0.91
Positive	3	3.2 ± 0.9		21.3 ± 5.2	
Perineural invasion					
Negative	15	3.2 ± 1.2	0.16	19.7 ± 5.9	0.10
Positive	7	3.9 ± 0.8		23.8 ± 3.6	

VSD, Vascular surface density; NVES, number of vessels per mm^2 stroma

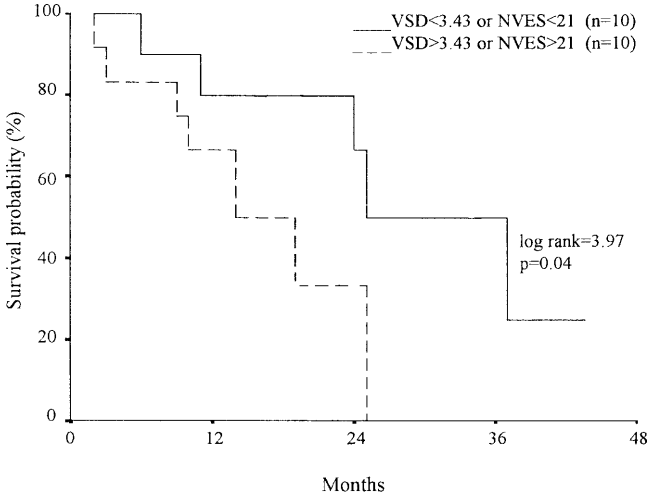

Fig. 6. Overall survival of patients who were stratified into two subgroups on either side of the mean value of vascular surface density (*VSD*) and number of vessels per mm^2 stroma (*NVES*) (cutoff values [means] of VSD and NVES were 3.43 and 21, respectively)

Table 2. Risk factors affecting overall survival using the multivariate Cox proportional hazard regression model

Variables	Hazard ratio	*P*
VSD (mm^{-1})[a]	1.88	0.039
NVES (mm^{-2})[b]	1.129	0.056

[a] Reduced model without NVES
[b] Reduced model without VSD

showed significant correlation with increased microvessel count ($P = 0.001$) and poorer differentiation ($P = 0.013$) (Table 1).

Discussion

Although considerable refinement has been achieved in the diagnosis, staging, and treatment of pancreatic cancer, few patients are truly cured.[1–5,7,11–13] Recently, considerable attention has been given to the prognostic significance of various clinicopathologic parameters that may reveal the biological basis of this disease and that may be employed to vary therapy according to the patient's predicted probability of survival.[2,11,13,26]

Angiogenesis, the outgrowth of new blood vessels from existing ones, is consistently reported to be a crucial step in tumor growth and progression. Different attempts have been undertaken to assess the degree of vascularization in solid tumors. Recent studies are affected by severe methodological drawbacks for the assessment of vessel counts on a virtually two-dimensional histological section influenced by coiling, tortuosity, and compression of vessels.[25] In our study, these methodological disadvantages were eliminated by using stereologic assessment of the angiogenesis.

Increased VSD and NVES showed significant association with high-grade pancreatic carcinomas, increased proliferative activity, and a tumor size of 3 cm or more. Multivariate regression analysis also indicated that tumor angiogenesis, especially measured as VSD, was an independent prognostic factor of survival in patients with pancreatic ductal adenocarcinoma. To our knowledge at present, there are two studies in the literature on vessel count in human pancreatic tumors which reveal contrary results. Ellis et al.[26] have reported no significant correlation between vessel count and severity of the disease. On the other hand, Ikeda et al.[27] have found that intratumoral microvessel density and vascular endothelial growth factor expression were independent prognostic factors in pancreatic cancer patients. On the basis of experiments on the induction of tumors in the pancreatic islets of transgenic mice, Hanahan and Folkman[9] observed that the transition from hyperplastic islets to neoplastic cells was preceded by angiogenic activity. Egawa et al.[28] and Kawarada et al.[29] reported that angiogenesis played an important role in tumor growth, and found that inhibition of angiogenesis was effective in suppressing the establishment and subsequent growth of hematogenous micrometastasis of pancreatic adenocarcinoma. These observations have further strengthened the concept that angiogenesis is also an early, critical, step in pancreatic carcinoma. Because the process of tumor growth and metastasis is complex and involves multiple events, tumor angiogenesis may not be the single overwhelming factor that determines the survival of patients with pancreatic cancer. Different proteins expressed by the tumor cells, including integrins and their receptors and growth factor receptors, and growth factors in the stroma, may contribute to the differences in tumor biology.[19]

The assessment of tumor cell kinetics may reflect tumor aggressiveness,[30] and, recently, high proliferative activity has been shown to correlate with poor prognosis in a variety of human malignancies.[31–33] The monoclonal antibody Ki-67 detects a nuclear antigen which is exclusively expressed in proliferating cells in G1, S, and G2 phases and mitosis, but not in G0. Hence, the Ki-67 antibody allows the immunohistochemical detection of cycling cells and its expression provides a direct measure of cell growth. Investigations addressing the relation between proliferative activity and prognosis in pancreatic cancer have yielded discordant results.[34–36] Although Ki-67 overexpression was significantly correlated with high neovascularization and tumor grade in our study, no association was found with patient survival and other clinicopathologic variables such as tumor size, nodal status, or perineural invasion.

The *p53* tumor suppressor gene maintains genomic stability by regulating normal responses to DNA damage and other forms of genotoxic stress.[9] It is suggested that the *p53* gene is also involved in the suppression of angiogenesis.[7,9] In pancreatic carcinoma, the *p53* gene is reported to be mutated in approximately 50% to 70% of tumors.[3,4,7] As in many other solid tumors, it is likely that pancreatic cancers depend on an extended vascular bed for tumor expansion. Mutant *p53* can enhance angiogenesis by stimulation of vascular endothelial growth factor expression.[12,26] Also, loss of functional *p53* protein may downregulate the synthesis of thrombospondin, an inhibitor of angiogenesis.[7,12,13] Our preliminary findings indicate that *p53* mutations are restricted to perpetuated tumors which represent the more locally advanced stages of pancreatic cancer. Although tumor size of 3 cm or more was mainly associated with *p53* overexpression, morphometric vascular measurements did not provide any evidence about the relation between *p53* protein overexpression and angiogenesis. Logical speculation suggests a pivotal role of *p53*-independent regulatory pathways in pancreatic tumorigenesis.

The K-*ras* gene, together with the H-*ras* and N-*ras* genes — the *ras*-oncogene family — are the most frequently mutated dominant oncogenes in human neoplasias.[37] K-*ras* mutations were the commonest genetic event described in pancreatic carcinoma and were found in up to 85% of advanced cases of ductal adenocarcinoma.[38] Moreover, inactivated *p53* was associated with the frequently activated K-*ras* oncogene.[39,40] It has also been shown that overexpression of H-ras oncoprotein correlates with the upregulation of type IV collagenase/gelatinase (MMP-9), an enzyme which is important in mediating basement membrane and extracellular matrix degradation in metastasis and modulating vascular endothelial growth factor-mediated angiogenesis.[41,42] In the present study, only 3 tumor tissues (13.6%) showed H-*ras* immunopositivity and they were in an early tumor stage (T_2N_0) with negative *p53* expression. H-*ras* immunopositivity did not show any significant correlation with clinicopathologic variables. This may indicate that H-*ras* could be a candidate to be studied in *p53* mutation-negative pancreatic carcinomas. However, it is noteworthy that a significant subgroup of pancreatic carcinomas do not harbor mutations in any of the three *ras* genes,[7] suggesting that another genetic pathway may be involved in human pancreatic tumorigenesis.

To summarize, we conclude that, in pancreatic adenocarcinoma, tumor angiogenesis is closely related to tumor growth and patient survival. Although the sample size and patient recruitment were limited and heterogeneous, our findings suggest that, in patients with pancreatic adenocarcinoma, increased neovascularization is a significant independent prognostic factor and highly predictive of poor final outcome. The high incidence of *p53* mutations and lack of H-*ras* genetic alterations in locally advanced pancreatic tumors is intriguing. It is likely that H-*ras* mutations may play a role in a *p53*-independent carcinogenetic pathway. Because of the heterogeneous nature of this tumor, it is hoped that further studies may delineate the separate subclones that have evolved along different neoplastic pathways in pancreatic carcinoma.

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