

High expression of both desmoplastic stroma and epithelial to mesenchymal transition markers associate with shorter survival in pancreatic ductal adenocarcinoma

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ABSTRACT

Desmoplastic stroma (DS) and the epithelial-to-mesenchymal transition (EMT) play a key role in pancreatic ductal adenocarcinoma (PDAC) progression. To date, however, the combined expression of DS and EMT markers, and their association with variations in survival within each clinical stage and degree of tumor differentiation is unknown. The purpose of this study was to investigate the association between expression of DS and EMT markers and survival variability in patients diagnosed with PDAC. We examined the expression levels of DS markers alpha smooth muscle actin (α -SMA), fibronectin, and vimentin, and the EMT markers epithelial cell adhesion molecule (EPCAM), pan-cytokeratin, and vimentin, by immunohistochemistry using a tissue microarray in a retrospective cohort of 25 patients with PDAC. The results were examined for association with survival by clinical stage and by degree of tumor differentiation. High expression of DS markers - α -SMA, fibronectin, and vimentin- was associated with decreased survival at intermediate and advanced clinical stages ($p=0.006-0.03$), as well as with both poorly and moderately differentiated tumor grades ($p=0.01-0.02$). Interestingly, the same pattern was observed for EMT markers, *i.e.*, EPCAM, pan-cytokeratin, and vimentin ($p=0.00008-0.03$). High expression of DS and EMT markers within each clinical stage and degree of tumor differentiation was associated with lower PDAC survival. Evaluation of these markers may have a prognostic impact on survival time variation in patients with PDAC.

Key words: Pancreatic ductal adenocarcinoma; desmoplastic stroma; epithelial-to-mesenchymal transition; clinical stage; degree of tumor differentiation; survival.

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Introduction

Pancreatic cancer is one of the neoplasms with unfavorable prognosis, ranking first in lethality rate worldwide.¹ Pancreatic ductal adenocarcinoma (PDAC) is the most frequent subtype, accounting for 85% of tumors of this organ.^{2,3} The American Joint Commission on Cancer (AJCC) tumor node metastasis (TNM) staging system is the standard diagnostic tool in which the disease process^{4,5} and treatment guidelines⁶ are outlined. The AJCC has also incorporated the degree of tumor differentiation (G) as a further criterion.⁷ Together with the TNM, the degree of tumor differentiation determines patient prognosis.⁶ The G criterion categorizes the tumor from well-differentiated (low grade) to undifferentiated (high grade); the more undifferentiated the tumor is, the more aggressive its biological behavior is. In PDAC, the histological grade is based on the extent of glandular differentiation. If more than 95% of a tumor is composed of glands, it is classified as well-differentiated; if 50-95% of the tumor is composed of glands, it is moderately differentiated; and if less than 50% of it is composed of glands, it is poorly differentiated.⁶ Several studies have shown that the degree of tumor differentiation of pancreatic cancer is a key indicator of survival after tumor resection,⁷⁻¹⁰ although both AJCC criteria fail to explain the variability in the survival of patients with PDAC. Thus, 80% of patients with PDAC are diagnosed at advanced clinical stages and with a worse prognosis^{2,11} and the 5-year overall survival rate is 9%.^{2,11,12} Furthermore, although the median survival for all clinical stages is 8 months,¹³ the reason why some patients survive up to 5 years,⁴ regardless of their clinical stages and degree of differentiation at diagnosis, remains unknown.^{4,10,13}

PDAC is characterized as a prominent desmoplastic stroma (DS) defined by fibrotic tissue with an abundant extracellular matrix around the tumor tissue.¹⁴⁻¹⁶ The cells that promote the development of the DS are cancer-associated fibroblasts (CAFs).¹⁷ CAFs are activated during inflammation and carcinogenesis, acquiring a myofibroblast-like morphology and expressing proteins such as alpha smooth muscle actin (α -SMA), fibronectin, and vimentin,^{18,19} in addition to accumulating an excess of extracellular matrix molecules, cytokines, chemokines and growth factors.^{12,16-18,20-27} The DS helps to form more aggressive tumors, with an increased capacity for proliferation, invasion, and metastasis, as well as resistance to chemotherapy and radiotherapy.^{12,17,28,29} Several *in vitro* and *in vivo* studies have demonstrated that PDAC tumor cells stimulate the proliferation, migration, activation, and secretion of extracellular matrix proteins of CAFs.^{15,20,30} An example of this crosstalk between tumor cells and stromal cells is TGF- β which is secreted by tumor cells and considered one of the most important and well-studied factors inducing CAF activation.¹² In turn, CAFs stimulate the proliferation, apoptosis, evasion, migration, invasion, and metastasis of PDAC tumor cells.^{14,20,23,31,32} As a result of these mutual cellular interactions, the process of epithelial-to-mesenchymal transition (EMT) of neoplastic cells is induced within the tumor microenvironment.^{19,21,22,29,33} During EMT, neoplastic cell epithelial markers such as the epithelial cell adhesion molecule (EPCAM)³⁴ and cytokeratins³³ are gradually lost, and mesenchymal markers such as vimentin^{33,35} are expressed. EMT is a reversible transdifferentiation process controlled by complex interactions between multiple signaling pathways such as TGF β , Wnt, and Notch, which converge to a network of specific transcription factors that convert cancer cells of epithelial differentiation into a more mesenchymal phenotypic state.³⁶ The expression of mesenchymal markers favors the development of an invasive phenotype in which the tumor cell increases its migration,²⁰ as shown in both *in vitro*^{21,37} and *in vivo*^{20,31} models. In fact, it has been shown that T β_4 , a peptide that regulate actin polymerization is pre-

dominantly expressed at the invasion front in colorectal tumor cells undergoing EMT, which suggest a role for T β_4 in invasion and metastasis.³⁸ Therefore, microenvironment dynamics promotes tumor growth and invasion^{29,39} and EMT might be helpful as a biomarker in PDAC diagnosis and to be considered as a new therapeutic target in the management of this disease.⁴⁰

The current prognostic stratification of PDAC based on the TNM and G criteria does not efficiently differentiate patients of different clinical stages who may survive for a period longer than the average time,⁴¹ including those at clinical stages III and IV.^{6,42} Consequently, new parameters must be included to help efficiently discriminate the survival of patients with PDAC within each clinical stage and tumor differentiation grade. Given that PDAC is characterized by an abundant DS and by the presence of EMT, biomarkers of both biological characteristics may be associated with PDAC aggressiveness and poor prognosis of patients with PDAC. Previous studies have associated the increased expression of DS markers, such as α -SMA^{43,44} or EMT markers such as EPCAM, cytokeratins, and vimentin,⁴⁵⁻⁴⁹ with a tendency for low survival among patients with PDAC. However, the combined expression of markers of both characteristics and their association with variations in survival within each clinical stage and degree of tumor differentiation remain unknown. In this context, we hypothesize that quantitative evaluation of the expression levels of DS and EMT markers may improve the current stratification and provide a highly accurate prognosis. Therefore, in this pilot study, we evaluated the expression of markers of both characteristics and analyzed their association with survival variability in patients diagnosed with PDAC.

Materials and Methods

Patients and samples

All patients in this study were diagnosed with PDAC and underwent pancreatic resection and biopsy at the Oncology Hospital, National Medical Center, Century XXI (Centro Médico Nacional Siglo XXI [CMN Siglo XXI]) and Mexican Institute of Social Security (Instituto Mexicano del Seguro Social [IMSS]), Mexico City. Since PDAC is not as frequent as other tumors in our hospital such as in other International Medical Centers,^{1,50} we decide to study these markers in a first approach, as a pilot study. It has been suggested that the minimal number of samples must be calculated according to data from Cochran's sample size formula⁵¹ based on i) a level of $\alpha = 0.001$; ii) a low margin of error = 3%; and iii) a low disease frequency = 0.0014. Thus, in our study 25 samples were collected and analyzed from 2007 to 2018. Our work is not the unique in the usage of reduced number of samples, in fact small cohorts of 7 and 10 patients have already been documented in other studies, respectively.^{52,53} Clinicopathological data were collected and included gender, age, stage T, stage N, stage M, TNM stages, degree of differentiation, overall survival, evolution time, the follow-up time of the first and the last medical consultation, the follow-up time of the disease onset at the last visit, desmoplasia, and type or surgery. Samples were fixed in formaldehyde and subsequently embedded in paraffin to prepare tissue microarrays (TMA).

The use of human samples was approved by the local Health Research Ethics Committee of the CMN Siglo XXI and IMSS and recorded under protocol number R-2016-3602-37.

Tissue microarrays

To minimize experimental variability and to ensure reproducible staining, TMA technology was applied using paraffin-

embedded tissues.⁵⁴ To corroborate the diagnosis of the pancreatic tumor samples, the hematoxylin and eosin stainings were re-evaluated by two expert pathologists, according to the 2010 World Health Organization classification. For the tumor samples, three replicates with a diameter of 1 mm were selected; these replicates were marked by our pathologist who selected areas with tumor tissue and desmoplastic stromal tissue for re-embedding into a recipient paraffin block using a tissue microarrayer (Chemicon International, Temecula, CA, USA). Once the TMAs were prepared, they were cut into serial 3- μ m sections. The microarray tissue slide contained approximately 75 cores corresponding to tumor samples from 25 patients, with three replicates each.

Immunohistochemistry

TMAs were stained with goat recombinant polyclonal anti- α -SMA antibody (1:250 dilution; Abcam, Cambridge, UK), rabbit recombinant polyclonal anti-fibronectin (1:1,000 dilution; Abcam), mouse recombinant monoclonal anti-vimentin (1:1,000 dilution; NSJ Bioreagents, San Diego, CA, USA), mouse recombinant monoclonal anti-EPCAM (1:500 dilution; BioLegend, San Diego, CA, USA), and mouse recombinant monoclonal anti-pan-cytokeratin (cocktail of epithelial cytokeratins CK 7, 8, 9, 17, 18, and 19; 1:500 dilution; NSJ Bioreagents) antibodies. The slides were dewaxed in an oven at 60 °C for 30 min and by an immediate washing in baths with detergent (EZ PREP 1 \times) for 5 min to remove the remaining paraffin from the slides. For antigen recovery, samples were subsequently immersed in a pH 6 citrate buffer (trisodium citrate dehydrate; Sigma-Aldrich, St. Louis, MO, USA) preheated for 5 min in a microwave oven. Then slides were heated for 10 min in a microwave pressure cooker (Nordic Ware, St. Louis Park, MN, USA) until temperature of 120°C was reached. Once the samples cooled down to room temperature, they were immersed in distilled water for 10 min and then immediately immersed in phosphate-buffered saline (PBS) (HyClone, Logan, UT, USA) with 10% Tween (Sigma-Aldrich) for 5 min. Subsequently, endogenous peroxidase and proteins (background staining) were blocked using Bloxall (Vector Laboratories, Burlingame, CA, USA) for 20 min and 2.5% normal horse serum (Vector Laboratories) for 20 min at room temperature. After carefully removing the horse serum, the samples were incubated with primary antibodies along with antibody diluent (Leyca Biosystems, Wetzlar, Germany) at 4°C overnight. The following day, primary antibodies were detected using the ImmPRESS[®] horseradish peroxidase (HRP) horse anti-goat, anti-rabbit, and anti-mouse IgG polymer detection kits (Vector Laboratories), for anti- α SMA, anti-fibronectin, and anti-vimentin, anti-EPCAM, and anti-pan-cytokeratin antibodies, respectively. All secondary antibodies were incubated for 30 min at room temperature. Once the secondary antibodies were washed, slides were covered in chromogen diaminobenzidine (Abcam) for 5 min, immediately washed twice in PBS (HyClone) to inhibit the chromogen reaction, and then immersed in distilled water for 5 min. Next, the slides were covered with Harris hematoxylin (Golden Bell, Guadalajara, Mexico) for 30 s and immediately washed with filtered water for 5 min. The slides were then treated with lithium carbonate (Golden Bell) and washed again in filtered water for 5 min. Isopropyl alcohol (ICR, Mexico City, Mexico) was used to dry the slides, and the samples were immediately mounted using rapid mounting medium (Sigma-Aldrich). Duplicates were made for each type of immunohistochemistry staining. Colon tissue from a human autopsy was used as a control for immunohistochemical markers in all stainings. In fact, epithelial and stromal cells of this tissue were used as negative controls for desmoplastic stroma and EMT markers, respectively.

Immunohistochemistry evaluation procedure

To avoid subjectivity in the immunohistochemistry staining evaluation, computer-assisted quantitative image analysis was performed using Aperio ePathology Solutions software (Leica Biosystems Imaging Inc., Wetzlar, Germany). Digital images of the slides were acquired using a ScanScope digital scanner (Aperio ePathology Solutions). The acquired images were observed and organized using the ImageScope viewer. The Aperio Spectrum/eSlide manager system (Aperio ePathology Solutions) was used to quantify the DS and EMT markers. The percentage of marker expression in pixels (positive staining area) was calculated using the V9 positive pixel count algorithm according to the immunohistochemical analysis of each marker.⁵⁵ A PDAC expert pathologist selected the tumor core (total expression for each antibody) and the areas in the TMA corresponding to desmoplastic stromal tissue and tumor tissue. During the selection of areas, artifacts (tissue folds) that could affect the analysis were ignored. The Aperio software was used to calculate the percentage of expression in pixels, which was classified as moderate pixel expression [number of positive pixels (Np)], strong pixel expression [number of strong positive pixels (Nsp)], negative pixel expression [number of negative pixels (Nn)], and total pixel expression, [number of total pixels, positive + negative (Nt)]. The following formula was used to calculate the percentage of expression in pixels: $Np + Nsp / Nt \times 100$.^{55,56} Values for each tumor core and each tissue area were averaged according to the number of samples obtained per patient (triplicates). The resulting quantification was the net number for each sample (only the cores containing at least 50% of the sample were included in the analysis).

Statistical analysis

To visually compare the patterns of protein expression percentages between each clinical stage, differentiation grade, and survival, a heatmap of the Z-score was constructed according to previous studies.^{52,57} We made a heatmap based on a hierarchical grouping of the expression levels of each marker using a data normalization of the Z distribution (difference between baseline minus mean between standard deviation) then we order it by clinical stage, degree of differentiation and survival. The values were classified according to the band of the Z score as high expression (pink to red colors) if the data for each marker were above average, and low expression (light to dark blue colors) if the data for each marker were below average. The average data for each antibody were marked in white, whereas data for each antibody that could not be obtained were marked in black.

SPSS ver. 21 software (IBM, Armonk, NY, USA) was used to perform statistical analyses. Chi square test was used to determine the association between clinical stage, degree of differentiation, survival and resectability (categorical parameters) and the expression of markers in tumor cores in desmoplastic stromal tissues and tumor tissues were classified as high and low expression, and the average of each marker was considered the cut-off point. Marker data was classified as categorical parameters: 0 (low expression) and 1 (high expression). Data were considered significant when the p value was <0.05.

Overall survival was calculated as the time elapsed from the date of pathology diagnosis to the date of death or the date of the last follow-up if the patient was still alive. Kaplan-Meier analysis was used to evaluate the relationship between the expression level of the studied proteins and the general survival time. The samples were classified as high and low expression, and the average of each marker was considered the cut-off point. In addition to the global expression of each protein, data were subclassified considering the clinical stage of the patients and the degree of differentiation. The Log-rank test was used to assess the importance of differences in

survival curves between samples with high or low expression of marker,⁵⁸ using the data analysis software Origin 8 (OriginLab, Northampton, MA, USA). Data were considered significant when the p-value was <0.05.

Results

Clinicopathological findings

Clinicopathological data were retrieved from a database generated from clinical records of the Medical Archive department of the Oncology Hospital. TNM stage was determined according to the TNM classification, which was determined from preoperative imaging findings, intraoperative findings, and postoperative pathological findings.⁵ Our study collected data on 15 male (60%) and 10 female (40%) PDAC patients, with a mean age at diagnosis of 63 years (ranging from 41 to 82 years). We found at the diagnostic time that most of the patients were classified in the advanced stage T and N, however most patients at stage M were found to correspond to M0. Clinical stage II was predominant in about 56% of the cases, while clinical stages III and IV corresponded to the remaining 44%. Regarding the degree of differentiation samples of most patients displayed a moderate degree of differentiation (64%). Evolution time ranged from 0 to 32 months, the follow-up time of the first and the last medical consultation ranged from 1 to 33 months, and the follow-up time of the disease onset at the last visit ranged from 0 to 39 months. In our study cohort, the mean overall survival was 5 months (range, 1-31 months). All cases were positive for desmoplasia, and the predominant type of surgery was Whipple procedure (44%). Clinicopathological data of the patients with PDAC are outlined in Table 1.

Expression of DS and EMT markers in PDAC samples

In this study, 75 samples from a cohort of 25 patients diagnosed with PDAC were analyzed to detect DS (α -SMA, fibronectin, and vimentin) and EMT (EPCAM, pan-cytokeratin, and vimentin) markers. These markers were selected because their expression has been suggested to affect the prognosis of patients with PDAC.¹⁷

The study markers were validated using a control sample of colon tissue (Figure 1). The α -SMA (Figure 1A), fibronectin (Figure 1E), and vimentin (Figure 1I) markers were expressed in the cytoplasm of stromal cells surrounding the colon epithelial glands (periglandular staining). The EPCAM (Figure 1M) and pan-cytokeratin (Figure 1Q) markers were expressed in the membrane and cytoplasm of colon epithelial cells (glandular staining), respectively. Tumor samples showed regions of DS in stromal tissue (Table 1). We did not find samples of clinical stage I in our study (Table 1); thus, the expression of DS markers was evaluated in stages II, III, and IV. The stromal markers α -SMA, fibronectin, and vimentin were located around the glands and tumor cells (perilesional staining) in the tumors (Figure 1, B-D; F-H; and J-L). Positive α -SMA staining was observed in 22 cases (88%), 13 of which were at clinical stage II (52%, Figure 1B), 4 at clinical stage III (16%, Figure 1C), and 5 at clinical stage IV (20%, Figure 1D). Positive α -SMA staining was found in 56% of cases with a moderate differentiation grade (Figure 1C) and in 32% of cases with a poor differentiation grade (Figures 1B and 1D). Positive fibronectin staining was observed in 21 cases (84%), 13 of which were at clinical stage II (52%, Figure 1F), 4 at clinical stage III (16%, Figure 1G), and 4 at clinical stage IV (16%, Figure 1H). Positive fibronectin staining was observed in 52% of cases with a moderate differentiation grade (Figure 1G) and in 32% of cases with a poor differentiation grade (Figures 1 F,H). The expression

of epithelial markers of EMT was also evaluated in tumor tissue (vimentin, pan-cytokeratin and EPCAM; Figure 1 J-L,N-P,R-T). Positive vimentin staining was found in 24 cases (96%), 13 of which were at clinical stage II (52%), 5 at clinical stage III (20%), and 6 at clinical stage IV (24%). Positive vimentin staining was found in 60% of cases with a moderate differentiation grade, but the staining was weak in most cases (*data not shown*) and negative in tumor glands (Figures 1 J,K). Positive vimentin staining was found in 36% of cases with a poor differentiation grade (Figure 1L). Both in DS and EMT, vimentin expression was observed in the same percentage for clinical stage and degree of differentiation.

Table 1. Clinicopathological features of a retrospective cohort of 25 patients with PDAC.

Parameter	Total (n=25)
Sex	25 (100%)
Male/female	15/10 (60%/40%)
Verage age at the time of surgery rank	63 (41-82)
Stage T	25 (100%)
T2	4 (16%)
T3	12 (48%)
T4	9 (36%)
Stage N	25 (100%)
N0	4 (16%)
N1	14 (56%)
N2	7 (28%)
Stage M	25 (100%)
M0	19 (76%)
pM1	6 (24%)
AJCC stage	25 (100%)
I	0 (0%)
II	14 (56%)
III	5 (20%)
IV	6 (24%)
Degree of differentiation	25 (100%)
Well differentiated	0 (0%)
Moderately differentiated	16 (64%)
Poorly differentiated	9 (36%)
Evolution time	24(96%)
Median evolution time in months (rank)	1 (0-32)
Follow-up time of the first and the last medical consultation	21 (84%)
Median follow-up time of the first and the last medical consultation (rank)	8 (1-33)
Follow-up time of the disease onset at the last visit	24 (96%)
Median follow-up time of the disease onset at the last visit (rank)	12 (0-39)
Survival	22 (88%)
Median survival in months (rank)	5 (1-31)
Desmoplasia	25 (100%)
Type of surgery	20 (80%)
Whipple	11 (44%)
Lape	3 (12%)
Subtotal pancreatectomy with splenectomy	1 (4%)
Multistructural resection	1 (4%)
Whipple and lape	2 (8%)
Pancreatectomy	2 (8%)

AJCC, American Joint Committee on Cancer.

Positive pan-cytokeratin staining was observed in 24 cases (96%), 14 of which were at clinical stage II (56%, Figure 1N), 5 at clinical stage III (20%, Figure 1O), and 5 at clinical stage IV (20%, Figure 1P). Positive pan-cytokeratin staining was found in 60% of cases with a moderate differentiation grade (Figure 1O) and in 36% of cases with a poor differentiation grade (Figures 1 N,P). Positive EPCAM staining was found in all 25 cases (100%), 14 of which were at clinical stage II (56%, Figure 1R), 5 at clinical stage III (20%, Figure 1S), and 6 at clinical stage IV (24%, Figure 1T). EPCAM-positive staining was found in 64% of cases with a moderate differentiation grade, marking tumor glands (Figure 1S), and in 36% of cases with a poor differentiation grade, showing a diffuse staining of the marker (Figure 1R). However, in some cases, EPCAM-negative areas were also observed in disaggregated tumor cells (Figure 1T).

The vimentin (Figure 1L), pan-cytokeratin (Figure 1P), and EPCAM (Figure 1T) photomicrographs derived from a serial case with a poor differentiation grade show positive staining for vimentin and pan-cytokeratin but not for EPCAM. All markers of

DS and EMT were positive in our cohort of study.

Clinicopathological variables and their association with DS and EMT markers

Once the expression of the markers was determined, we decided to analyze if there was an association between the DS and EMT with clinicopathological variables. For this purpose, we first identified if there were differences in the expression levels of each marker. For this evaluation, a visual multivariate analysis was performed, and a heatmap was produced for the z-scores (Figure 2). This method evidenced the expression heterogeneity of each study marker for the 25 cases of our cohort included AJCC stage, degree of differentiation and survival (Figure 2). For this reason, each marker was separated using its average expression in pixels as a reference and we classified each marker as either low or high expression in pixels. High expression of vimentin in tumor core was associated with clinical stages III and IV ($p=0.041$). High expression of α -SMA in tumor core ($p=0.033$), EPCAM ($p=0.030$) and pan-cytokeratin ($p=0.050$) in tumor tissue were associated

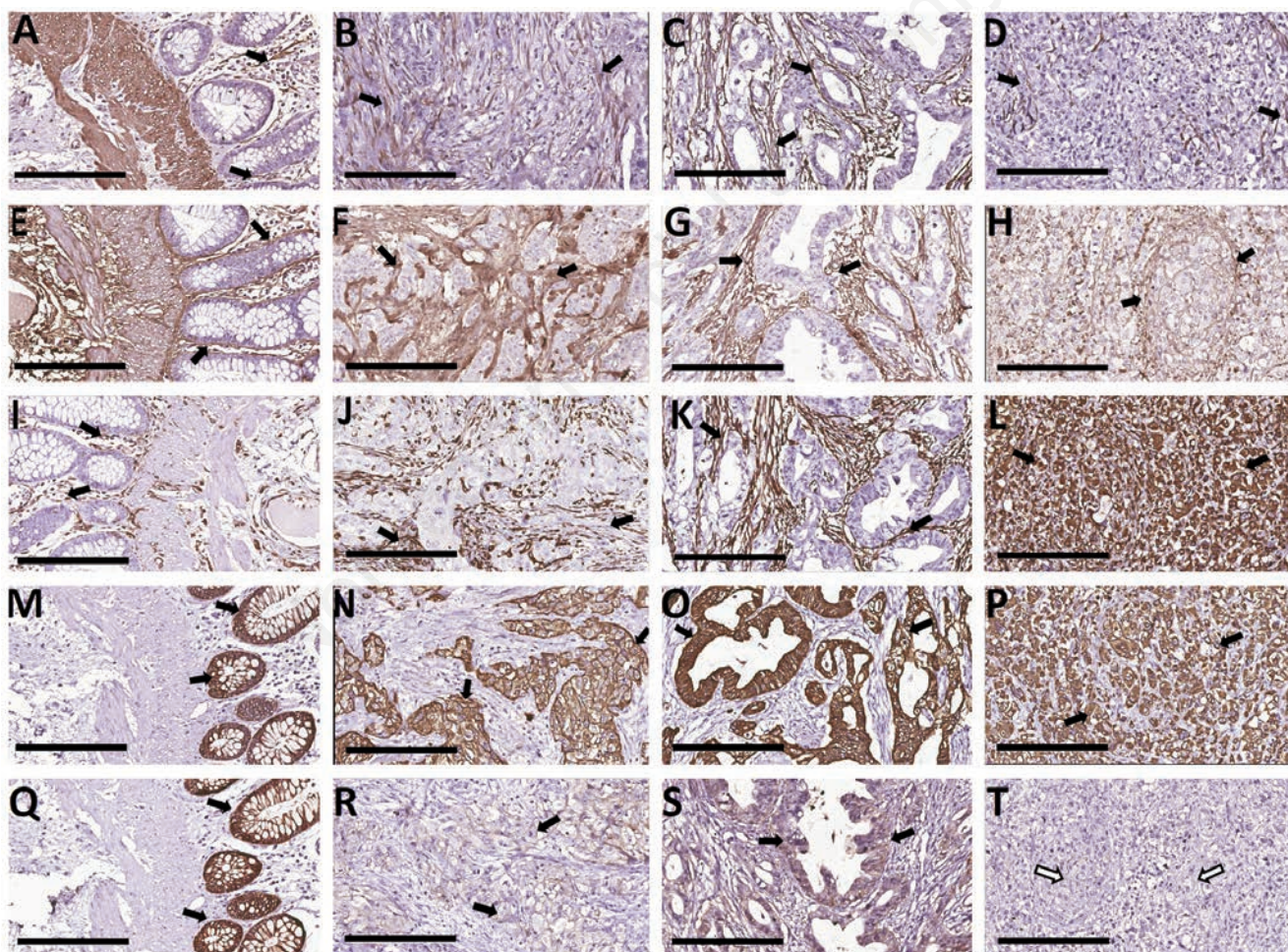


Figure 1. Immunohistochemical analysis of DS and EMT markers in control colon and PDAC tissues. Representative microphotographs of control colon tissues (A-Q). Periglandular staining of α -SMA-positive stromal cells (A). Periglandular staining of fibronectin-positive stromal cells (E). Periglandular staining of vimentin-positive stromal cells (I). Epithelial glands staining positive for EPCAM (M). Epithelial glands staining positive for pan-cytokeratin (Q). Black arrows indicate positive staining in stroma (A-I); Black arrows indicate positive staining in glands (M,Q). Representative cases of samples from patients with PDAC (B-T). Clinical stages II (B-R), III (C-S), and IV (D-T). Black arrows indicate perilesional α -SMA (B-D), fibronectin (F-H), and vimentin (J-K) staining in tumor stroma. Black arrows indicate vimentin (L), pan-cytokeratin (N-P), and EPCAM (R-S) staining in tumor cells. White arrows indicate negative EPCAM staining in tumor cells (T). All stains were counterstained using hematoxylin. Magnification: 20 \times . Scale bars: 200 μ m.

with a lower survival corresponding to less than 5 months. Low expression of fibronectin in tumor core was associated with a positive resectability ($p=0.026$). High expression of α -SMA in stromal tissue was associated with a poor differentiation grade ($p=0.035$). These results are summarized in Tables 2 and 3.

High expression levels of DS and EMT markers are associated with decreased survival as a function of clinical stage and degree of differentiation

We performed survival analysis using the current prognostic stratification (Figure 3 A,B) and found no statistically significant association between survival and clinical stage ($p=0.54$) and between survival and degree of differentiation ($p=0.40$). Subsequently, we decided to identify subgroups for predicting a lower survival rate for different clinical stages and degree of differentiation according to the expression of DS and EMT markers in tumor core, stromal and tumor tissue. Based on our previous multivariate analysis used with clinicopathological variables, we classified each marker as displaying either low or high expression in color-coded pixels for each clinical stage and degree of differentiation. High expression levels of α -SMA ($p=0.03618$) at clinical stage II and fibronectin ($p=0.01554$) at clinical stages III and IV in stromal tissue were associated with lower survival (Figure 4 A,B). High expression of vimentin at clinical stages II ($p=0.00683$), III and IV ($p=0.03735$) in stromal tissue was associated with lower survival

(Figure 4 C,D). High expression of pan-cytokeratin ($p=0.0393$) at clinical stage II was associated with lower survival (Figure 4E). High expression of pan-cytokeratin ($p=0.00014$) in tumor tissue and vimentin ($p=0.03735$) in tumor core were associated with lower survival at clinical stages III and IV (Figure 4 F,G). High expression levels of fibronectin and α -SMA were associated with a lower survival for moderate (α -SMA $p=0.02$) and poor (fibronectin $p=0.01$) differentiation grades in stromal tissue (Figure 4 H,I). High expression levels of vimentin ($p=0.012$) in stromal tissue were associated with lower survival for moderate differentiation grade (Figure 4J). High expression levels of pan-cytokeratin and EPCAM were associated with a lower survival for poor differentiation grade (pan-cytokeratin $p=0.00185$, EPCAM $p=0.000085$) in tumor tissue (Figure 4 K,L). High expression levels of pan-cytokeratin ($p=0.00977$) in tumor core were associated with lower survival for moderate differentiation grade (Figure 4M).

Discussion

In this study, we found that high expression levels of DS markers, such as α -SMA, fibronectin, and vimentin, were associated with lower patient survival at intermediate and advanced clinical stages, as well as with poorly and moderately differentiated grades. Interestingly, the same patterns were observed when analyzing the

Table 2. Association of clinicopathological features with markers on tumor core.

Clinicopathological variable	Markers on tumor core														
	α SMA			Fibronectin			Vimentin			EPCAM			Pan-cytokeratin		
	High	Low	p	High	Low	p	High	Low	p	High	Low	p	High	Low	p
Stage II	7	6	0.548	6	8	0.145	4	9	0.041	4	10	0.678	7	7	0.628
Stage III and IV	6	3		6	2		8	3		4	7		4	6	
Moderately differentiated	8	6	0.806	8	6	0.746	6	9	0.206	4	12	0.317	6	9	0.459
Poorly differentiated	5	3		4	4		6	3		4	5		5	4	
Survival time <5 months	9	1	0.033	6	4	0.845	7	4	0.528	5	6	0.17	5	6	0.801
Survival time >5 months	4	5		5	4		5	5		2	9		4	6	
Negative resectability	5	1	0.394	5	0	0.026	7	1	0.061	3	5	0.55	1	6	0.061
Positive resectability	7	4		5	7		5	6		3	9		7	5	

Table 3. Association of clinicopathological features with markers on stromal and tumor tissue.

Clinicopathological variable	Markers on stromal and tumor tissue																	
	α SMA (stromal)			Fibronectin (stromal)			Vimentin (stromal)			EPCAM (tumor)			Pan-cytokeratin (tumor)			Vimentin (tumor)		
	High	Low	p	High	Low	p	High	Low	p	High	Low	p	High	Low	p	High	Low	p
Stage II	6	7	0.937	7	6	0.466	6	7	0.188	5	9	0.622	7	7	1	3	10	0.247
Stage III and IV	4	5		3	5		8	3		5	6		5	5		5	6	
Moderately differentiated	4	10	0.035	5	8	0.284	7	8	0.134	6	10	0.734	7	8	0.673	3	12	0.074
Poorly differentiated	6	2		5	3		7	2		4	5		5	4		5	4	
Survival time <5 months	6	4	0.498	5	5	0.28	8	3	0.284	7	4	0.03	8	3	0.05	3	8	0.284
Survival time >5 months	4	5		2	6		5	5		2	9		3	7		5	5	
Negative resectability	2	4	0.232	2	3	0.889	5	3	0.96	5	3	0.094	5	2	0.109	4	4	0.552
Positive resectability	7	4		4	7		7	4		3	9		4	8		4	7	

EMT markers vimentin, pan-cytokeratin, and EPCAM. To our knowledge, this is the first study to establish an association between the expression of DS and EMT markers and survival by clinical stage and degree of differentiation, suggesting that their evaluation might be of help in estimating the survival time in patients with PDAC.

In our study cohort, a key issue is that vimentin has started to be evaluated as a DS marker in patients with PDAC,⁵⁹ because it has mainly been described as an EMT marker,^{48,60,61} however, we detected its expression in stromal tissue surrounding tumor glands,

in line with previous observations.⁶² In contrast to other studies which have reported the expression of pan-cytokeratin in tumor buddings,^{46,63} we detected pan-cytokeratin expression in glands and scattered tumor cells surrounded by DS in PDAC samples. Also, we observed a weak or even null EPCAM expression in areas of disaggregated tumor cells, which could also be found in EMT. Similar results have been described in *in vitro* cultures of breast tumor cells, which also showed decreased EPCAM expression associated with the EMT process.³⁴ In our study, vimentin was mainly expressed in disaggregated tumor cells. Therefore, the

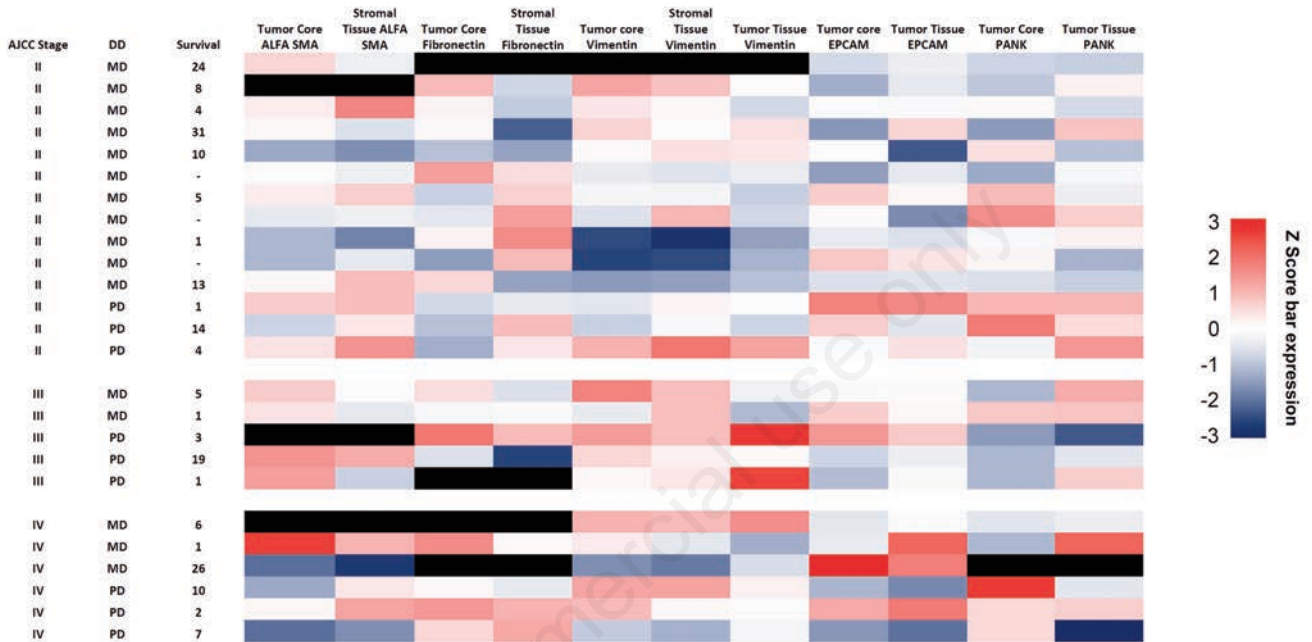


Figure 2. Heatmap of the Z-scores. Low staining levels are indicated by values ranging from -1 to -3 (blue color). High staining levels are indicated by values ranging from 1 to 3 (red color). Cells without values are indicated in black, and those that correspond to the average expression levels are indicated in white. Degree of differentiation (DD): moderately differentiated (MD) and poorly differentiated (PD). Survival is indicated in months.

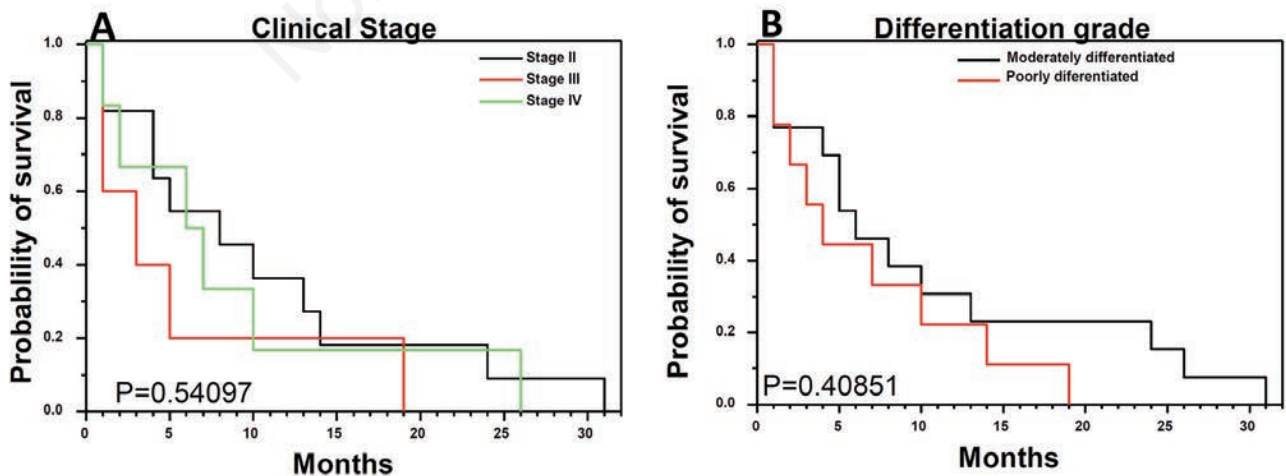


Figure 3. Current prognostic stratification and survival of patients with PDAC. Kaplan-Meier analysis of the overall survival of patients and clinical stage. Samples are marked in black for clinical stage II, in red for clinical stage III, and in green for clinical stage IV (A). Kaplan-Meier analysis of the overall survival of patients and differentiation grade. Samples with a moderate differentiation grade are marked in black, and samples with a poor differentiation grade are marked in red (B).

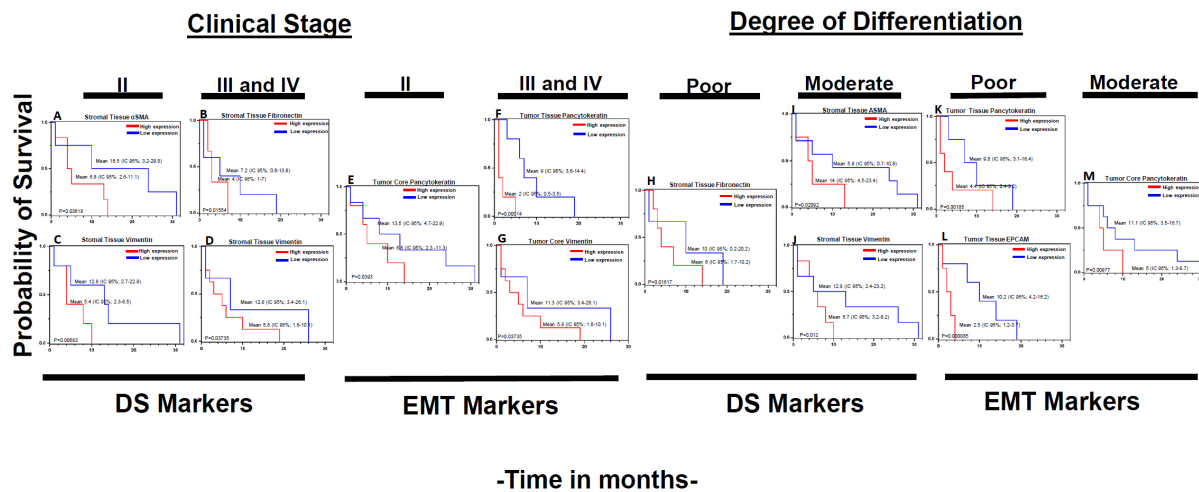


Figure 4. Association of DS markers and EMT with survival by clinical stage and degree of differentiation in PDAC. The expression of DS and EMT markers was quantified and associated with the overall survival of patients in a Kaplan-Meier analysis by clinical stage (A-G) and degree of differentiation (H-M). Survival analysis of α -SMA (n=10; A), vimentin (n=10; C) in stromal tissue and pan-cytokeratin in tumor core (n=11; E) were identified in clinical stage II. Survival analysis of fibronectin (n=8; B), vimentin in stromal tissue (n=11; D), pan-cytokeratin in tumor tissue (n=10; F) and vimentin in tumor core (n=11; G) were identified in clinical stages III and IV. Survival analysis of α -SMA (n=13; I), vimentin (n=15; J) in stromal tissue and pan-cytokeratin in tumor core (n=14; M) were identified in moderate degrees of differentiation. Survival analysis of fibronectin (n=8; H) in stromal tissue, pan-cytokeratin (n=9; K) and EPCAM (n=9; L) in tumor tissue were identified in poor degrees of differentiation. For clinical stage, samples with low expression are marked in blue, and samples with high expression are marked in red. Similarly, mean survival values and 95% confidence intervals (CI) are shown, and the p-values of the Log-rank test are shown.

close contact with DS suggests that in PDAC, these cells could be more easily released into the circulation in groups or individually.^{36,63,64} This is the first study to jointly assess the expression of two epithelial markers (pan-cytokeratin and EPCAM) and one mesenchymal marker (vimentin) by immunohistochemistry in PDAC and to report the association between high expression of vimentin with advanced clinical stage (III and IV). This dual interaction could explain why these types of tumors are more aggressive at advanced stages. Furthermore, the association of pan-cytokeratin with lower survival rates has never been reported, so far studies have only evaluated the expression of this marker as an identifier of tumor budding, which in turn is associated with a lower survival rate.⁴⁶ On the other hand, α -SMA has never been reported associated with a less degree of differentiation which involves the importance of DS in the development of more aggressive tumors and EMT, as reported in *in vitro* studies.^{14,20,23,29,31} The low expression of fibronectin was associated with a positive resectability, which implies that a minor quantity of DS could facilitate surgical intervention, because this stromal marker is part of the fibrotic tissue secreted by stromal cells that promote the development of the DS,^{16,39,65} which occupies more than 80% of the tumor volume.¹⁷ This association is reported for the first time.

According to the current prognostic stratification based on clinical stage (TNM) and degree of differentiation (G), in our study, these parameters were not associated with the survival of patients diagnosed with PDAC, in line with other published studies, albeit with a higher number of participants;⁶⁵ yet these studies contrast with others that incorporated a higher number of patients.^{4,6} Despite these differences, in each clinical stage and in even advanced stages, heterogeneity has been observed in the survival time of patients, which is not explained by the current prognostic stratification.^{41,42}

When choosing a panel of DS and EMT antibodies for each patient, we found in our study cohort that all markers were heterogeneously expressed, as shown by the heatmap, thus indicating the

presence of high- and low-expression subgroups. Our quantitative analysis allowed us to observe that the higher expression of all markers defined a shorter survival for each clinical stage and degree of differentiation, in contrast to other studies in which these markers were individually evaluated in terms of overall survival of patients with PDAC.^{44,45,48,65} Some preliminary findings from a semi-quantitative analysis performed in patients with PDAC indicates that EMT markers may be associated with a shorter survival in clinical stages III and IV^{40,45} and stromal markers could be associated with a shorter survival in clinical stages II;⁴⁴ in fact in our cohort of Mexican patients with this subtype of pancreatic cancer this behavior is observed. However, when classifying clinical stages and differentiation grades by subgroups, our study cohort was reduced; hence, further studies should be conducted using an increased sample size. Furthermore, markers such as T β ₄, which has been suggested plays an important role in the process of EMT in colorectal cancer³⁸ and the roles of different subsets of CAFs within the stroma¹² could also be evaluated.

In conclusion, we suggest that including DS and EMT markers in prognostic stratification may improve our understanding of different survival times of patients with PDAC, regardless of the clinical stage and degree of differentiation at which they are diagnosed, thereby improving current prognostic stratification.

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