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Original Article

TRAIL receptors are expressed in both malignant and stromal cells in pancreatic ductal adenocarcinoma

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Abstract: This study assesses the expression of all TNF-related apoptosis-inducing ligand (TRAIL) receptors in pancreatic ductal adenocarcinoma (PDAC) tumor tissue. We aimed to include TRAIL receptor expression as an inclusion parameter in a future clinical study using a TRAIL-based therapy approach for PDAC patients. Considering the emerging influence of PDAC desmoplastic stroma on the efficacy of anti-PDAC therapies, this analysis was extended to tumor stromal cells. Additionally, we performed PDAC stroma characterization. Our retrospective cohort study (N=50) included patients with histologically confirmed PDAC who underwent surgery. The expression of TRAIL receptors (DR4, DR5, DcR1, DcR2, and OPG) in tumor and stromal cells was evaluated by immunohistochemistry (IHC). The amount of tumor stroma was assessed by anti-vimentin IHC and Mallory's trichrome staining. The prognostic impact was determined by the univariate Cox proportional hazards regression model. An extensive expression of functional receptors DR4 and DR5 and a variable expression of decoy receptors were detected in PDAC tumor and stromal cells. Functional receptors were detected also in metastatic tumor and stromal cells. A poor prognosis was associated with low or absent expression of decoy receptors in tumor cells of primary PDAC. After assessing that almost 80% of tumor mass was composed of stroma, we correlated a cellular-dense stroma in primary PDAC with reduced relapse-free survival. We demonstrated that TRAIL functional receptors are widely expressed in PDAC, representing a promising target for TRAIL-based therapies. Further, we demonstrated that a low expression of DcR1 and the absence of OPG in tumor cells, as well as a cellular-dense tumor stroma, could negatively impact the prognosis of PDAC patients.

Keywords: TRAIL receptors, pancreatic adenocarcinoma, stroma characterization

Introduction

Pancreatic ductal adenocarcinoma (PDAC) accounts for the vast majority of pancreatic cancer and is the fourth leading cause of cancer death in both men and women in western countries [1]. However, recent trends in incidence and mortality suggest that PDAC might soon become the most fatal cancer. In the European Union (EU), pancreatic cancer represents a significant illness not showing favorable trends in recent years [2], thus reflecting the inadequacy of our current treatment options. With a five-year relative survival rate life expectancy of approximately nine percent [3], the lowest rate among solid tumors, and an incidence that nearly matches mortality, PDAC is a challenging disease that compels advances in research. Late diagnosis, aggressive behavior (i.e., early invasion and metastasization) and presence of abundant stroma are likely concause of the high rate of relapse even after curative surgery [4, 5], account for the inauspicious previsions of this disease.

The human PDAC microenvironment is characterized by consistent hyperplasia, called desmoplasia, of the stroma surrounding neoplastic cells. This hyperplastic tissue is composed of both cellular (cancer-associated fibroblasts [CAF], pancreatic stellate cells, immune cells) and non-cellular components (extracellular matrix mainly composed of collagen fibers) [6-8]. These elements facilitate crosstalk with cancer cells, promoting their survival, resulting in a poor prognostic impact on PDAC patients [9, 10]. Knudsen and colleagues identified three different stromal subtypes according to the number of cancer-associated fibroblasts and the quantity of extracellular matrix [9]. A high level of intratumor stroma relative to the neoplastic component represents a physical barrier to the tumor, hampering neoplastic growth and dissemination and providing a beneficial effect for the patient [11]. However, stromal abundance in PDAC restricts tumor vasculature, compromising the delivery of chemotherapy to the tumor [11, 12].

Given these unique features of PDAC, an urgent need exists for novel therapeutic compounds, such as the TNF-related apoptosis-inducing ligand (TRAIL) and focused interventions, capable of going beyond the traditional purely chemotherapeutic approach. *TRAIL* is a physiologically produced protein involved in various biological processes, including the reaction against infectious, autoimmune, and neoplastic diseases. The first and better-characterized function consists of inducing apoptosis in malignant cells through a p53-independent mechanism while sparing non-transformed cells [13-15]. The biological effects of TRAIL are exerted through the activation of two signaling (functional) receptors that contain a conserved death domain motif: Death receptor 4 (DR4) and Death receptor 5 (DR5). The complexity of TRAIL's receptor system is, however, unprecedented. In addition to the two functional DRs, three other receptors bind to TRAIL but are incapable of transmitting an apoptosis signal, therefore acting as decoys: Decoy receptor 1 (DcR1) lacks the intracellular death domain; Decoy receptor 2 (DcR2) has a truncated, nonfunctional death domain; Osteoprotegerin (OPG) is a soluble receptor that prevents TRAIL-DR4/DR5 interaction through the binding of soluble TRAIL [16].

Recently, TRAIL-based antitumor approaches have emerged as promising alternative treatment options. Recombinant human forms of TRAIL, followed by TRAIL-receptor agonist monoclonal antibodies and their combination with other components, have been challenged in pre-clinical and clinical trials, showing good tolerability but limited therapeutic effects due to several factors (e.g., extremely short half-life, poor tumor-targeting efficacy, resistance to TRAIL monotherapy) [17-20]. For these reasons and to overcome limitations, researchers have moved toward the possibility of exploiting cell-based gene therapeutic approaches, generating stably modified mesenchymal stromal cells (AD-MSC) to obtain cellular vehicles for a targeted and constant TRAIL delivery system [21-27].

TRAIL receptors (TRAIL-R) are highly expressed in a variety of cancers, including PDAC, suggesting that these tumors could be treated by antineoplastic therapies that exploit TRAIL, possibly improving PDAC patient outcomes. Although expression of TRAIL-R is not the only determinant of response to the TRAIL apoptotic effect, TRAIL-based compounds may prove effective in a TRAIL-R expression-dependent fashion in individual tumors [28]. Therefore, the expression of TRAIL-R in PDAC specimens may represent an important criterion to identify patients that might benefit from a TRAIL-based therapy. In addition, given the crucial role of the PDAC stromal compartment in influencing the efficacy of treatment, the potential susceptibility of these cells to TRAIL apoptotic impact could be relevant to patient outcomes. Therefore, we conducted immunohistochemical (IHC) analyses to evaluate death receptor (DR4, DR5) and decoy receptor (DcR1, DcR2, OPG) expression in a cohort of fifty surgical specimens of PDAC, considering not only the tumor compartment but also the stroma. Given its pivotal role in the aggressive behavior of PDAC, we further characterized the stroma compartment both quantitatively and qualitatively, confirming and supporting data from previous studies.

Materials and methods

Ethics statement

Histological analyses on archived PDAC samples were conducted after authorization by the

ethics committee of the University of Modena and Reggio Emilia (CE 298/14).

Case identification and selection

Patients with histologically confirmed PDAC, who underwent surgery for pancreatic cancer at the Department of Surgery of the University Hospital of Modena and the Public Hospital of Baggiovara between 2001 and 2015, were considered for our retrospective cohort study. Formalin-fixed and paraffin-embedded (FFPE) PDAC samples were retrieved from the archive of the Institute of Pathology of the University Hospital of Modena. We collected all clinicopathological data from pathological reports and electronic medical records present in the database of the Cancer Center of Modena. Only deceased patients were included in the study. Since TRAIL-R expression is affected by the administration of chemotherapy and radiotherapy [29-31], patients who received neoadjuvant treatments (chemotherapy and/or radiotherapy) were excluded. Pathological tumor-node-metastasis (pTNM) staging was determined according to the 8th edition of the American Joint Committee on Cancer [32]. Tumor Grading was determined according to the 5th edition of the WHO Classification of Digestive System Tumors [33]. In addition, for a preliminary evaluation of functional TRAIL-R on PDAC metastases, we selected 8 biopsies of confirmed liver metastases from PDAC patients. Similarly to primary PDAC cohort, patients did not receive chemotherapy and/or radiotherapy before the biopsy.

Tissue selection and histology

For each selected patient, a preliminary screening of the most representative PDAC tissue portion was performed retrospectively on hematoxylin and eosin (H&E) stained tissue prepared for routine diagnostic examination from pancreatectomy and collected in the archive of the Pathological Anatomy Unit of the University Modena Hospital. The corresponding FFPE PDAC samples were then cut to obtain 6 μm sections. For the IHC evaluation of TRAIL-R expression, the following antibodies (all purchased from Abcam, Cambridge, UK) were employed: rabbit polyclonal anti-DR4 (dilution 1:50), rabbit polyclonal anti-DR5 (dilution 1:100), rabbit monoclonal anti-DcR1 [EPR6162] (HRP) (dilution 1:1000), rabbit monoclonal anti-DcR2 [EPR3588(2)] (dilution 1:200), rabbit polyclonal anti-Osteoprotegerin (dilution 1:200). For PDAC stromal cell quantification, a rabbit monoclonal anti-Vimentin [EPR3776] antibody (Abcam, dilution 1:2000) was used. We performed the IHC reactions with the Ultraview Universal DAB detection kit and the fully automated IHC slide staining instrument BenchMark XT (Roche, Basel, CH). Negative controls omitting primary antibodies were run simultaneously. IHC slides were digitalized using the Axiocam ICc3 microscope (Zeiss, Oberkochen, Germany). Collagen fibers in the stromal extracellular matrix were stained using Mallory's trichrome staining kit (BioOptica, Milan, Italy).

Tissue evaluation

After IHC, we assessed TRAIL receptor expression on stained tissue by visual analysis in double (Nikon E400 microscope, magnification 20× and 40×). For tumor tissue, a semi-quantitative scoring system was developed: score 0 (*negative staining*), score 1 (*weakly-to-moderately positive staining*), or score 2 (*strongly positive staining*). TRAIL-R localization in tumor cells was also evaluated in stained samples. Due to the soluble nature of OPG, its cellular localization was not considered relevant and was therefore not investigated.

For TRAIL-R analysis on stromal tissue, we determined negative or positive staining for each slide.

For stroma quantification, the PDAC stromal cellular compartment was defined as the vimentin-stained slide portion with neoplastic cells at all edges. The identified PDAC stromal area was digitally scanned at 100× magnification (at least 20 fields per sample). The percentage of positive-stained area in each image was then calculated using the Color Deconvolution plugin in ImageJ, and the mean stromal cell amount for each sample was evaluated. Mallory's trichrome stained slides were digitally scanned at 63× (10 fields per sample) and quantified by the Image analysis plugin of Zen software (Zeiss). For the stromal architecture analysis, we developed a semi-quantitative scoring system according to the prevalent PDAC stromal cell density in each sample: Low Density (loose or moderate stromal cell density with a prevalence of sample stroma occupied

Table 1. Summary of clinicopathological features

Feature	Patient Count	
	n	%
Age (median, y)		
≤68	23	46%
>68	27	54%
Gender		
Female	20	40%
Male	30	60%
Stage		
Early	32	64%
Locally advanced/Metastatic	18	36%
Grading		
Well, moderate	29	58%
Poor	21	42%
Margin status		
Negative	37	74%
Positive	13	26%
Postoperative CT		
No	20	40%
Yes	30	60%
Postoperative RT		
No	40	80%
Yes	10	20%
Relapse		
No	10	20%
Yes	40	80%
Tumor location		
Head	43	86%
Body	6	12%
Tail	1	2%
Tumor size, median (cm)		
≤3.4	29	58%
>3.4	21	42%
Vascular invasion		
Yes	35	60%
No	15	30%
Perineural infiltration		
Yes	44	88%
No	6	12%
Surrounding non-neoplastic parenchyma		
PanIN1	8	16%
PanIN2	18	36%
PanIN3	16	32%
Chronic Pancreatitis	24	48%
Fibrosis	1	2%
None	1	2%
Site of relapse		
Liver	23	46%
Peritoneum	11	22%
Lung	4	8%
Lymph nodes	10	20%
Loco-regional	10	20%
Other	4	8%

Abbreviations: CT = Chemotherapy; RT = Radiotherapy.

by extracellular matrix) and High Density (dense cell stroma with a low presence of extracellular matrix).

Statistical analysis

Descriptive statistics of patients included in the study were calculated; categorical data were reported as absolute and percentage frequencies, and numerical variables as median and range. When appropriate, numerical variables were divided into two classes based on the median observed value. We assessed the overall survival (OS) and relapse-free survival (RFS) as the time in days between surgery and death or relapse, respectively. The association of the parameters of interest with OS and RFS was measured with a univariate Cox proportional hazards regression model. The results were reported as Hazard Ratio (HR) with 95% confidence interval (95% CI) and *P*-value. Moreover, Kaplan-Meier survival curves were calculated. All analyses were performed with R 3.6.0 statistical software (The R Foundation for Statistical Computing, Wien) at the $P<0.05$ significance level.

Results

Patient selection and clinicopathological features

Fifty consecutive patients with histologically confirmed PDAC who underwent surgery for pancreatic cancer at the Department of Surgery of the University Hospital of Modena and the Public Hospital of Baggiovara between 2001 and 2015 were identified. A summary of the clinicopathological features of our patients' cohort is shown in **Table 1**. The median patient age at the time of surgery was 68 years (range 42-84), and the majority of patients were male (60%). Locally advanced or metastatic cancer was observed in 36% of patients, whereas a poor grading (i.e. G3) was observed in 42% of patients.

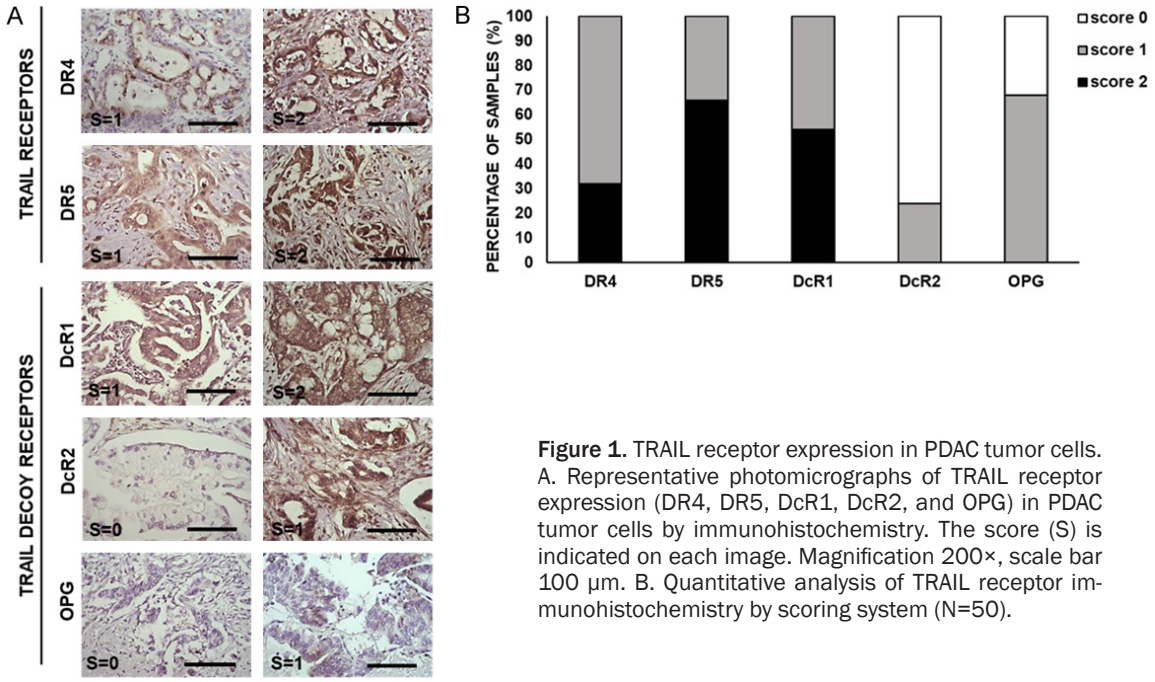

Figure 1. TRAIL receptor expression in PDAC tumor cells. A. Representative photomicrographs of TRAIL receptor expression (DR4, DR5, DcR1, DcR2, and OPG) in PDAC tumor cells by immunohistochemistry. The score (S) is indicated on each image. Magnification 200×, scale bar 100 μm. B. Quantitative analysis of TRAIL receptor immunohistochemistry by scoring system (N=50).

During a total follow-up time of 72.5 person-years, 40 patients (80.0%) experienced a relapse, and all patients (100.0%) died. The incidence rate for relapse was equal to 88.2 cases per 100 person-years, and the median relapse time was 0.9 years (95% CI=0.6, 1.2). The mortality rate was equal to 69.0 deaths per 100 person-years and the median OS time was 1.2 years (95% CI=1.0, 1.7). Other collected clinicopathological features of the experimental cohort are summarized in **Table 1**.

TRAIL receptor expression and localization in PDAC tumor tissue

We collected histological data on the expression of TRAIL functional receptors (DR4 and DR5) and TRAIL decoy receptors (DcR1, DcR2, and OPG) in tumor cells from PDAC specimens taken from 50 affected patients (**Figure 1A**). As shown in **Figure 1B**, 100% of PDAC specimens from enrolled patients displayed histological positivity in neoplastic cells for both TRAIL functional receptors DR4 and DR5. DR4 was expressed as score 1 and score 2 in 68% (n=34) and 32% (n=16) of patients, respectively. DR5 was expressed as score 1 in 34% (n=17) and as score 2 in 66% (n=33) of patients. A varying expression of TRAIL decoy receptors was observed in tumor cells (**Figure 1B**). DcR1 was expressed in all analyzed PDAC samples, with intensity ranging between score 1 (46%, n=23) and score 2 (54%, n=27). Focusing on DcR2 expression, 76% (n=38) of PDAC samples were negative, and positive specimens had a weak intensity (score 1: 24%; n=12). For OPG, 32% (n=16) of PDAC samples were negative, and positive specimens displayed a weak intensity (score 1: 68%; n=34).

We observed different staining localizations of TRAIL receptors in tumor cells (**Figure 2**). Staining involved both the plasmatic membrane and the cytoplasm, or just the latter (**Figure 2A**). Additionally, nuclear positivity was detected in tumor cells after anti-DR5 IHC (**Figure 1A**). DR4 was present in the malignant cell cytoplasm of 68% of PDAC samples and distributed in both cytoplasm and membrane in 32% of samples; DR5 was located primarily inside the cytoplasm (88% of samples) (**Figure 2B**). For decoy receptors, DcR1 was detected both within the cytoplasm and on the cell surface (cytoplasm expression: 48%; membrane and cytoplasm expression: 52%), while DcR2 staining was mainly localized in the cytoplasm (**Figure 2B**).

TRAIL receptors expression in PDAC stroma

We also evaluated the expression of TRAIL receptors in the stromal compartment of PDAC

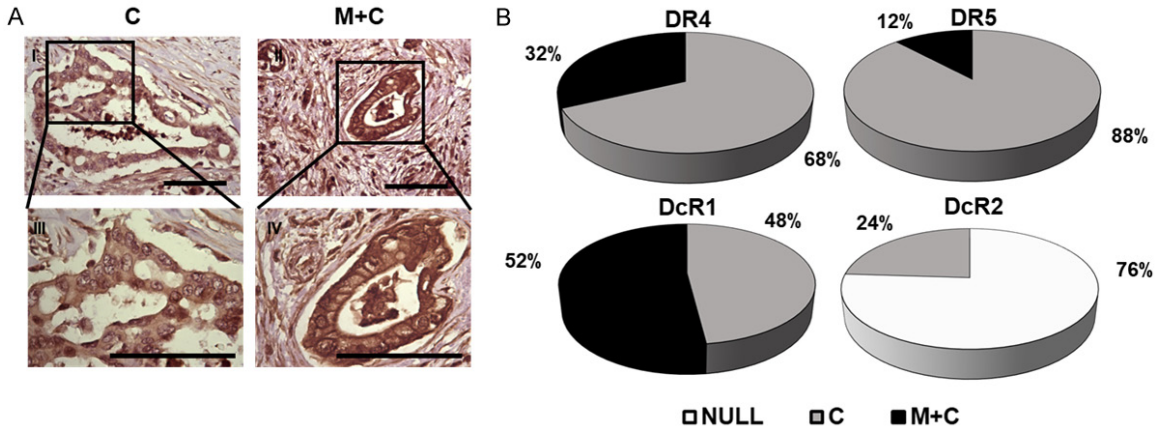

Figure 2. TRAIL receptor localization in tumor cells of PDAC patients. A. Representative microphotographs of TRAIL receptor localization by immunohistochemistry. C, cytoplasmic distribution of the receptor; M+C, membrane and cytoplasmic distribution. I and II, magnification 200×; III and IV, magnification 400×. Scale bar 100 μm. B. Percentages of distribution of TRAIL receptors in tumor cells of PDAC samples.

samples, defined as the stromal area delimited by neoplastic cells on all sides (**Figure 3**). Because the varying stromal density among samples could alter the perceived staining intensity and localization, we focused on staining negativity or positivity (**Figure 3A**). All PDAC samples stained positive for DR5 in the stromal tissue. In contrast, the majority of samples (84%, n= 42) were negative for DR4 (**Figure 3B**). Concerning decoy receptors, stromal cells expressed DcR1 and DcR2 in 100% of samples, while the soluble receptor OPG was detected in the stroma of 38% (n=19) of the samples.

TRAIL receptor expression in PDAC metastases

Beside the cohort of 50 primary PDAC samples, we extended TRAIL-R analysis also on liver metastases from PDAC (**Figure 4A** and **4B**). In this case, we decided to evaluate only functional receptors, because a low amount of tumor material was available. All selected specimens (100%, n=8) displayed the expression of DR4 and DR5 in tumor cells, with a weak staining intensity (score 1) for DR4 and a strong staining intensity (score 2) for DR5. Looking at TRAIL-R localization in metastatic cells, DR4 was detected in the cytoplasm of tumor cells, while DR5 was expressed both in the nucleus and in the cytoplasm (**Figure 4A**).

The amount of stroma was far less abundant in respect to primary PDAC, but we were able to assess that stromal cells in all analyzed metastatic samples were negative for DR4, while all specimens expressed DR5 (100%, n=8) (**Figure 4B**).

PDAC stroma characterization

We then studied cellular (anti-Vimentin IHC) and non-cellular (Mallory's trichrome staining) components for stroma characterization. Upon visual analysis, PDAC stroma was more abundant relative to the stroma in the normal surrounding tissue (**Figure 5A**). Based on the prevalent cell density in the tumor stroma, we dichotomized samples into two patterns of tumor stroma: in 60% of tumors, a low density of stromal cells (Low Density) was observed, while 40% of samples displayed a high cellular density (High Density) in PDAC stroma (**Figure 5A**). The histological quantification of the total stromal amount revealed that 45% of pathological tissue was composed of stromal elements (i.e., CAF and, depending on the inflammatory grade in each sample, immune cells), and 32.5% were composed of collagen fibers (**Figure 5B**). The histological evaluation of the total stromal amount was performed also separately in Low Density and High Density groups, but we did not observe differences in the mean values of vimentin and collagen expression between the two groups of samples (data not shown).

Prognostic correlation

We assessed the impact of our histological data regarding the expression of TRAIL-R and

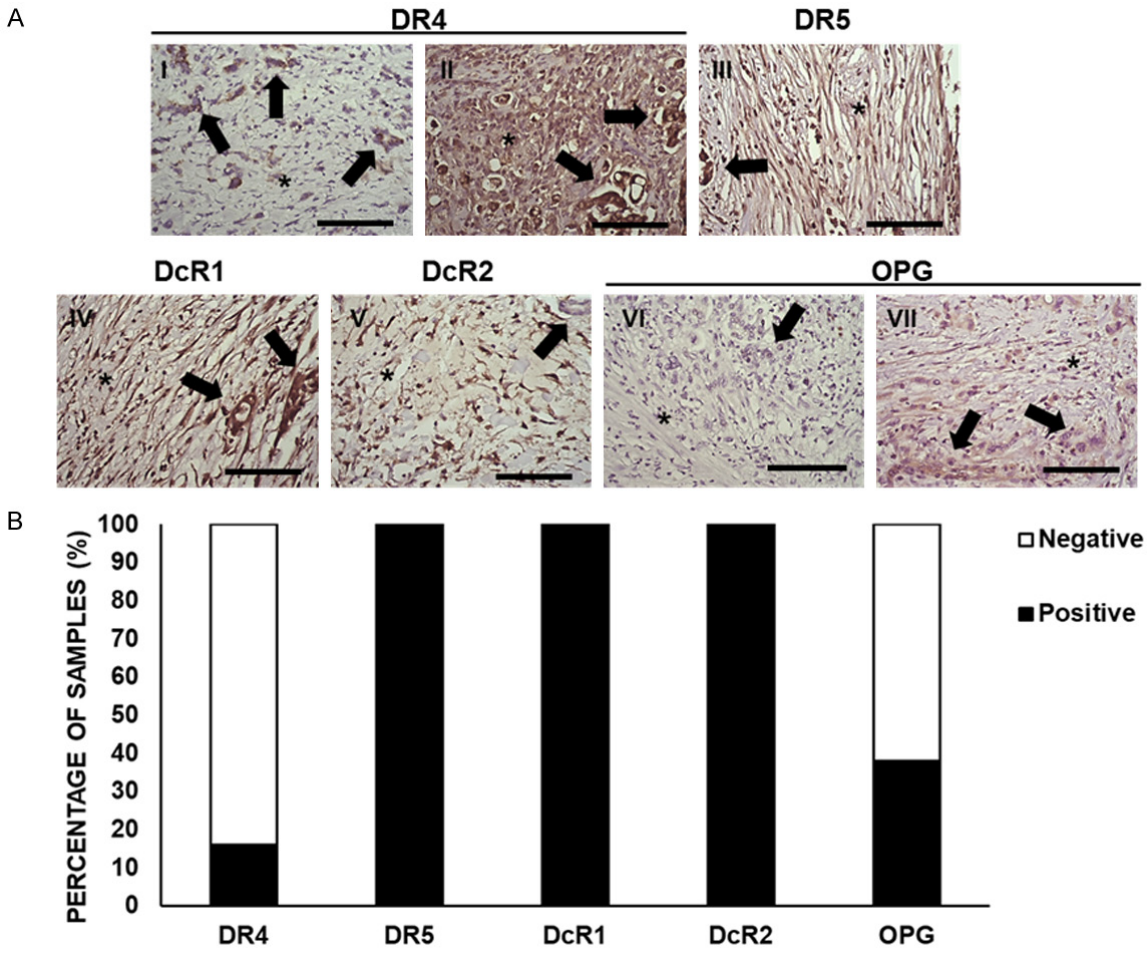

Figure 3. TRAIL receptor expression in PDAC stroma. A. Representative photomicrographs of TRAIL receptor expression (DR4, DR5, DcR1, DcR2, and OPG) in PDAC stromal cells by immunohistochemistry. Black arrows indicate tumor cells, and asterisks indicate stromal areas. I, negative staining for DR4; II, positive staining for DR4; III, positive staining for DR5; IV, positive staining for DcR1; V, positive staining for DcR2; VI, negative staining for OPG; VII, positive staining for OPG. Magnification 200×, scale bar 100 μm. B. Quantitative analysis of TRAIL receptor expression in tumor stroma (N=50).

stroma characterization on the prognosis of PDAC patients using a Cox univariate analysis. Focusing on TRAIL-R expression in tumor cells, we determined that a low expression of DcR1 (score 1) correlated with a worse prognosis (worse OS) for patients than a higher expression (score 2) of this receptor (HR=0.47; 95% CI=0.26, 0.86; P=0.013) (**Figure 6** and **Table 2**). Similarly, patients with no tumor expression of OPG (score 0) had a lower life expectancy than patients with OPG-positive tumors (score 1) (HR=0.53; 95% CI=0.28, 0.98; P=0.043). No other differences in OS or RFS were observed on TRAIL-R expression in tumor cells (**Table 2**). Furthermore, no significant correlations were observed between TRAIL-R expression by tumor stromal cells and the prognosis of PDAC patients. TRAIL-R localization in tumor cells did not affect prognosis (**Table 2**).

Cox analysis revealed that high cellular density in tumor stroma (High Density) correlated significantly with a higher risk of tumor relapse compared with patients with lower stromal density (Low Density) (HR=1.95; 95% CI=1.02, 3.70; P=0.043) (**Figure 6** and **Table 2**). Conversely, no statistical correlations were detected between patients' prognosis and collagen levels.

To provide a critical interpretation of our results, we assessed whether a correlation existed between the statistically significant parameters and the clinicopathological variables that

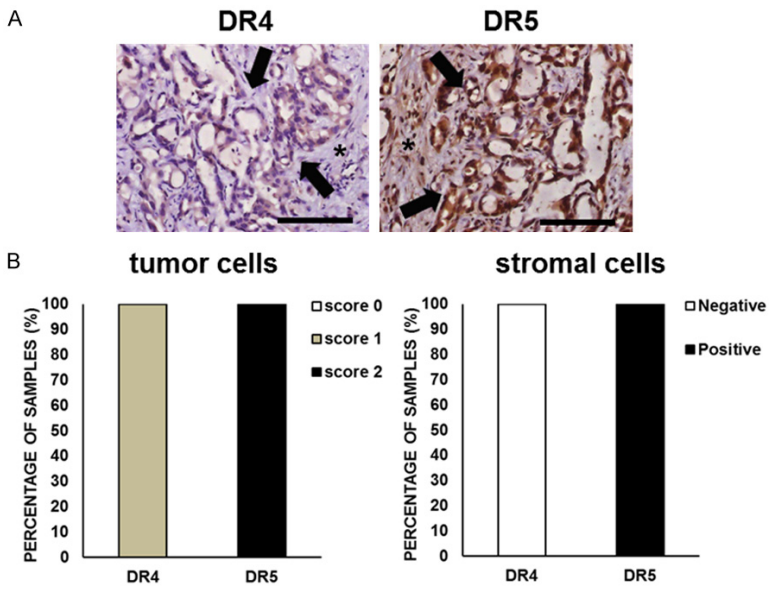

Figure 4. TRAIL functional receptor expression in PDAC metastases. A. Representative photomicrographs of TRAIL functional receptor expression (DR4 and DR5) in tumor and stromal cells of liver metastases from PDAC by immunohistochemistry. Black arrows indicate representative tumor cells, and asterisks indicate stromal areas. Magnification 200×, scale bar 100 μm. B. Quantitative analysis of TRAIL functional receptor expression in tumor cells and stromal cells of liver metastases from PDAC (N=8).

influence OS and RFS. A summary of this analysis is reported in **Table 3**.

Remarkably, 61% of tumors with a low (score 1) expression of DcR1, 69% of tumors with no (score 0) expression of OPG and 65% of tumors with a high stromal cell density (High Density) were of an early stage (stage I or II). Moreover, 78% of tumors with a low expression of DcR1, 88% of tumors with no expression of OPG and 85% of tumors with a high stromal cell density had a negative margin status after resection, which is associated with a favorable prognosis after surgery [34]. Almost half (48%) of samples with low expression of DcR1 and 75% of samples with high stromal cell density were associated with well/moderate grading (G1/G2), while the majority (69%) of those with no expression of OPG were poorly differentiated (G3).

Discussion

Here we provide a comprehensive evaluation of the expression of all TRAIL receptors (DR4, DR5, DcR1, DcR2, and OPG) in tumor and stromal cells from patients affected by PDAC.

Despite the relatively small size, our study population was highly representative of the PDAC patient population in terms of clinicopathological characteristics and relapse and survival parameters. The median age at diagnosis (68 years) corresponded to the median age at diagnosis in the general population, which is approximately 71 years, and the majority of tumors were localized in the head of the pancreas [35, 36]. Further, the high prevalence of vascular invasion and perineural infiltration was consistent with the inauspicious characteristics of this recalcitrant tumor. The incident rate for relapse, 88.2%, was in line with the high postoperative recurrence rate of approximately 75-92% [37, 38], and the median sample post-resection OS of 14 months remained in the range of 11-20 months [39].

Thus far, very few studies have focused on TRAIL-R expression in PDAC, and none of them has considered all five receptors [28, 40, 41]. Here, we described an extensive expression of DR4 and DR5 in PDAC tumor cells. In particular, based on staining intensity, DR5 expression seems higher than that of DR4. Moreover, we observed a variable expression, often with low intensity, of decoy receptors in the tumor compartment. Sanlioglu and colleagues tested 34 PDAC patients for the presence of DR4, DR5, DcR1, and DcR2, discovering a higher expression level of DR4 and DcR2 in tumor tissue of PDAC patients relative to healthy pancreatic tissue [41]. Gallmeier et al. (2013) found that 77% and 99% of PDAC specimens were positive for DR4 and DR5, respectively, while 52% and 69% of specimens were positive for DcR1 and DcR2, respectively. In addition, this study correlated low DR5 expression with reduced OS in PDAC patients with no nodal metastasis after surgery (pN0) [28]. Finally, Gundlach et al. (2018) recently published work recognized high expression of DR4 by tumor cells as a favorable prognostic marker in PDAC [40].

Unlike the studies cited above, we did not extend the analysis to normal pancreatic ductal

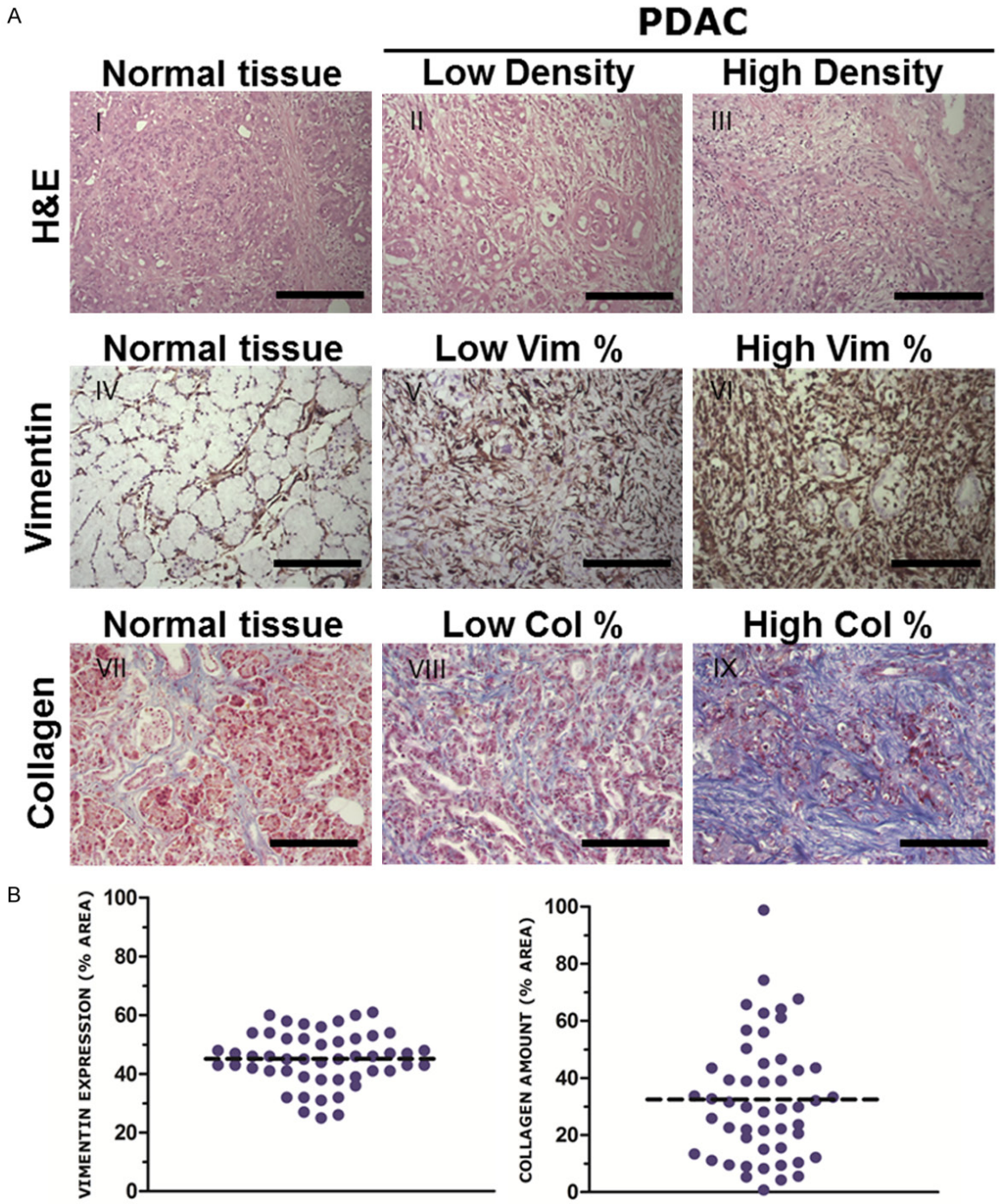

Figure 5. PDAC stroma characterization and quantification. A. Representative images of the normal surrounding pancreatic and tumor stromal architecture in PDAC samples. Hematoxylin and eosin staining (H&E, I-III), anti-vimentin IHC (IV-VI) staining, and Mallory's trichrome (VII-IX) staining are shown. Magnification 100×, scale bar 200 μm. B. Vimentin and Collagen quantification (N=50). Each circle represents the mean percentage of positive pixels in a sample. The dashed line indicates the total mean value.

cells because healthy surrounding tissue was rare and confined to the external areas, rendering it prone to non-specific IHC staining. We chose tissue sections with a high PDAC quantity to be as representative as possible of the whole tumor mass for our purposes. Similar to the studies discussed above, our results demonstrated a relevant expression of TRAIL func-

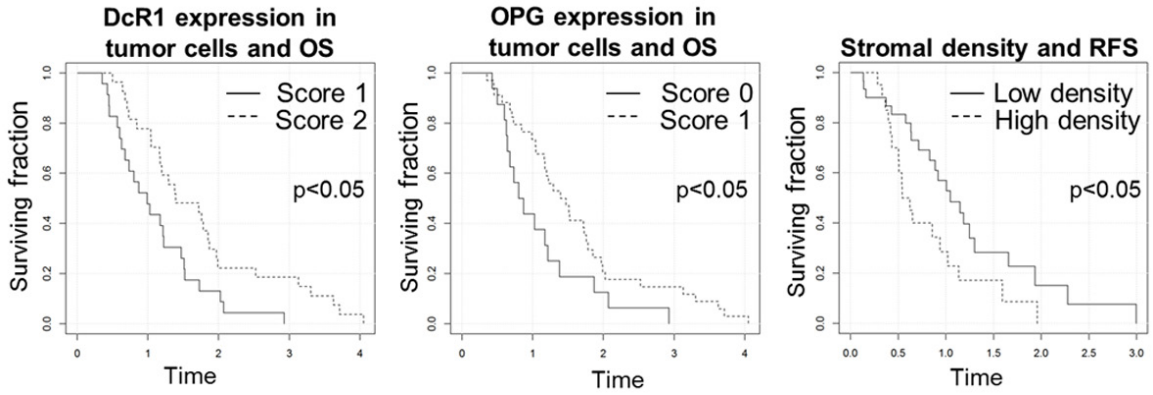

Figure 6. Statistical correlations. Kaplan-Meier curves of PDAC patients according to DcR1 expression in tumor cells, OPG expression in tumor cells and stromal cellular density. OS, Overall survival; RFS, Relapse-free survival.

Table 2. Univariate overall survival and relapse-free survival analysis

Variable			Overall Survival			Relapse Free Survival		
			HR	95% CI	P	HR	95% CI	P
Tumor	DR4	score 2 vs 1	1.138	0.61; 2.11	0.680	1.233	0.62; 2.43	0.546
		localization M+C vs C	1.019	0.55; 1.88	0.951	1.271	0.65; 2.47	0.481
	DR5	score 2 vs 1	0.632	0.34; 1.16	0.138	0.936	0.48; 1.84	0.847
		localization M+C vs C	1.196	0.50; 2.84	0.685	1.241	0.51; 2.99	0.632
	DcR1	score 2 vs 1	0.472	0.26; 0.86	0.013	0.784	0.41; 1.51	0.465
		localization M+C vs C	0.851	0.48; 1.51	0.580	1.058	0.56; 1.99	0.862
	DcR2	score 1 vs 0	0.927	0.47; 1.83	0.828	1.149	0.54; 2.45	0.720
		localization C vs NULL	0.927	0.47; 1.83	0.828	1.149	0.54; 2.45	0.720
	OPG	score 1 vs 0	0.528	0.28; 0.98	0.043	0.634	0.32; 1.27	0.199
Stroma	DR4	score 1 vs 0	0.812	0.36; 1.68	0.528	1.248	0.57; 2.74	0.581
	OPG	score 1 vs 0	0.906	0.50; 1.63	0.743	0.934	0.49; 1.80	0.838
	Stromal cell density	high vs low	1.504	0.83; 2.71	0.175	1.945	1.02; 3.70	0.043
	Vimentin quantification	high (≥45.5%) vs low (<45.5%)	0.750	0.43; 1.32	0.319	0.857	0.45; 1.63	0.638
	Collagen quantification	high (≥29.9%) vs low (<29.9%)	0.796	0.45; 1.41	0.432	1.127	0.59; 2.14	0.714

Abbreviations: HR = Hazard Ratio; 95% CI = 95% confidence interval; P = *P*-value.

tional receptors in primary human PDAC samples [28, 41]. However, in contrast to the studies conducted by Gallmeier (2013) and Gundlach (2018), we did not observe any correlation between the expression of TRAIL-R and patient prognosis. However, we attributed a negative prognostic impact to tumors with low DcR1 expression or no OPG expression [28, 40].

Despite the paucity of PDAC-specific data, the correlation between TRAIL-R expression and patient prognosis has been evaluated in other cancers. In breast cancer, a higher expression of DR4 was observed in well-differentiated tumors and correlated positively with markers of a better prognosis (hormone receptor status, Bcl-2, negative nodal status). On the contrary, DR5 and DcR2 expression correlated negatively with prognosis and overall survival of patients [42]. Two studies focusing on colon cancer showed a high expression of DR4 and DR5 in the majority of analyzed tumors and associated DR4, but in opposite ways, with prognosis [43, 44]. In hepatocellular carcinoma, high expression of DR4 and moderate expression of DR5 were detected, and the loss of either of these receptors significantly worsened patients' five-year survival rate [45]. In another study, samples of non-small-cell lung cancers expressed DR5, and 67% showed a high expression of this receptor, correlating with poorly differentiated tumors and lower overall survival [46].

Moreover, Vigneswaran et al. (2007) found a high expression of DR5 in oral squamous cell carcinoma correlated with larger tumors [47].

Table 3. Association of statistically significant parameters and clinicopathological variables with potential impact on OS and RFS

Clinicopathological features	DcR1 score 1 (n=23)	OPG score 0 (n=16)	Stromal density HIGH (n=20)
Stage			
Early	14 (61%)	11 (69%)	13 (65%)
Locally advanced/Metastatic	9 (39%)	5 (31%)	7 (35%)
Margin status			
Negative	18 (78%)	14 (88%)	17 (85%)
Positive	5 (22%)	2 (12%)	3 (15%)
Grading			
Well/Moderate	11 (48%)	5 (31%)	15 (75%)
Poor	12 (52%)	11 (69%)	5 (25%)
Age			
≤68	9 (39%)	6 (38%)	10 (50%)
>68	14 (61%)	10 (62%)	10 (50%)

Finally, in prostate cancer, a higher expression of DcR2 correlated strongly with PSA recurrence, a high Gleason score, and decreased survival [48]. In summary, numerous studies have demonstrated that TRAIL-R is highly expressed in various cancers, including PDAC, suggesting that these tumors could be treated by antitumoral therapies that exploit TRAIL-R ligands and possibly improve patient survival. However, the prognostic significance of TRAIL-R has always concerned functional receptors and not decoy receptors; exceptions include the studies by Ganten et al. (2009) on breast cancer and Koksal et al. (2008) in prostate cancer [42, 48].

Focusing on TRAIL-R localization, we observed that death receptors were often located in the cytoplasm of tumor cells rather than on the cell membrane. Gundlach et al. (2018) highlighted the difficulty in differentiating plasma membrane from cytoplasm when staining DR4 using IHC and chose not to include the cell membrane in the staining evaluation [40]. In accordance with our results, which included the nuclear positivity observed in DR5-stained tumor cells, emerging evidence has shown that DR4 and DR5 are mainly expressed intracellularly rather than in the plasma membrane of malignant cells [40, 49, 50]. This evidence could reflect the presence of an intracellular reservoir of receptors able to translocate to the cell membrane [40]. In addition, it could represent the internalization of activated TRAIL-R as part of the TRAIL-induced signal pathway [51]. Other explanations may relate to nuclear non-apoptotic functions of DR4 and DR5, including a tumor-promoting effect [49, 52], or may reflect a defense strategy against TRAIL-induced apoptosis [45, 53].

Building on previous research, we decided to analyze the expression of TRAIL-R in the stromal compartment. Indeed, the stroma is highly abundant in PDAC, and the presence of TRAIL-R on stromal cells could positively or negatively influence the delivery and effect of TRAIL-based therapy to tumor cells. In our cohort, all samples expressed at least one functional receptor (DR5) on stromal cells. Hence, a TRAIL-based treatment could theoretically target the stromal compartment, and we recommend adding this approach to anti-stromal therapies currently under investigation [54].

As a preliminary study, we evaluated the expression of functional TRAIL-R also on tumor and stromal cells in liver metastases of a small cohort of PDAC patients. Both functional receptors were expressed in metastatic tumor cells, while in stromal cells we were able to detect only DR5. Despite the low number of analyzed metastatic samples, we demonstrated that functional TRAIL-R is expressed in a similar way both in primary tumor and liver metastases. At our knowledge, no other studies reported data on TRAIL-R expression in PDAC metastases, and further studies on a larger cohort of metastatic patients are needed.

Besides the analysis of TRAIL-R expression, we characterized PDAC stromal tissue, both qualitatively and quantitatively, to compare our results with previous studies. In PDAC, neoplastic cells are surrounded by a consistent amount of stroma composed of cellular and acellular elements [9]. In our study, we demonstrated that approximately 80% of PDAC consists of stroma. This result is in line with data published by other groups [55-59]. Morphologically, we observed two distinct subtypes of stroma based on stromal cell density. The presence of high cell density stroma correlated with a higher risk

of developing tumor relapse. In order to avoid coarse and confounding bias, we checked for the possible association between the high cell density stroma parameter and clinicopathological variables that have a well-known potential impact on RFS. Interestingly, the majority of tumors with a high cell density stroma pattern displayed a negative margin status, generally associated with a low risk of relapse, and G1/G2 (well/moderate) tumor grading, This suggests that, beside the clinical features commonly associated with an unfavorable prognosis, the High Density stroma pattern may represents a further promising parameter to take into account in the next future when estimating the risk of relapse of PDAC. Certainly, the limited number of samples warrants further confirmation and validation to increase the robustness of the finding. Notably, a similar result was obtained by Knudsen et al. in 2017, who evaluated hematoxylin and eosin-stained sections and associated highly cellular PDAC stroma (defined as *immature*) with a worse prognosis than tumors containing low cellular stroma levels (defined as *mature*) [9].

The presence of a dense stromal cell population may facilitate crosstalk between neoplastic and stromal cells, stimulating tumor progression. The strong link between these components has been widely confirmed by in vitro studies [60, 61]. Conversely, a low stromal cell density and abundant extra-cellular matrix may interfere with secretome exchange between tumor and stromal cells. Indeed, many studies have shown a positive correlation with prognosis in patients with collagen-rich PDAC [5, 55, 62]. Interestingly, we did not observe a correlation between the total stromal cell amount (vimentin quantification) or total extracellular matrix (collagen quantification) and prognosis. Hence, it seems that a high stromal cell density, regardless of absolute stromal cells or collagen quantity, may represent an aggressive phenotype of malignant cells. Analyzing stroma quantifications separately in Low Density and High Density stromal cells groups, we observed very similar mean values in both vimentin and collagen quantification. Vimentin and collagen amounts were determined by extensive analyses of all tumor stroma areas in each sample in order to obtain quantitative and reliable data on the total stromal amount. The two groups displayed very similar mean values in both vimentin and collagen quantification despite to the stromal density. This could be due to the fact that stromal cell density represents a qualitative parameter that defines the spatial organization of stromal cells in PDAC stroma rather than effective cell number. Therefore, it is not necessarily related to the low/high absolute amount of stroma in each sample, but it is mainly dependent to the closeness of the stromal cells in the tumor area.

In conclusion, intending to employ TRAIL-R expression as an inclusion parameter in clinical studies focused on a TRAIL-based treatment approach, we assessed the expression of TRAIL receptors and TRAIL decoy receptors in PDAC, showing that this tumor represents a promising target. Notably, a poor prognosis was associated with low or absent expression of decoy receptors in tumor cells. We also confirmed the negative impact of a cellular-dense stroma on PDAC patient prognosis, but further studies are required to better characterize PDAC stromal tissue in this patient population.

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Disclosure of conflict of interest

MD and GGr hold patents in the field of cell and gene therapy and declare a consultancy role, research funding, and stock ownership with Rigenerand Srl. MCS declares stock ownership with Rigenerand Srl. MDa and MCS are employees of Rigenerand Srl. The other authors do not declare any competing interests.

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