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From Cell Biology to Tissue Engineering

The expression of death receptor systems TRAIL-R1/-R2/-R4, CD95 and TNF-R1 and their cognate ligands in pancreatic ductal adenocarcinoma

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Summary. The expression of five members of the TNF receptor superfamily and two of their ligands in human pancreatic ductal adenocarcinoma were investigated by immunohistochemistry. 41 patients with histologically confirmed ductal carcinoma of the pancreas were enrolled in this study in order (i) to compare the individual TNFR-SF expression and their ligands in PDAC-cells and (ii) to investigate their correlation with survival data. All patients had undergone pancreaticoduodenectomy and were staged as pT3N1M0. Immunostaining was done on FFPE tissue sections of the tumor tissue, using antibodies directed against TRAIL-Receptor-1, -2 and -4, TRAIL, CD95, TNF-Receptor-1 and TNF-α. The intensity and quantity of immunostaining were evaluated separately for tumor cell cytoplasm and tumor cell nucleus. Immunostaining results were correlated with each other and with patient survival. All proteins were found to be expressed in the majority of the tumor cells. The expression (i) of the following members of TNFR-SF and their ligands correlated with each other: TNF-Receptor-1 and TNFα (cytoplasmatic scores, p=0.001), TNF-Receptor 1 and TRAIL (nuclear antigen expression p=0.005 and the main score p=0.001, which contains the overall intracellular antigen expression), TNF-Receptor 1 and CD95 (main score, p=0.001), TRAIL-Receptor-1 and TRAIL-Receptor-2 (nuclear parameters, p=0.023), TRAIL-Receptor-4 and TRAIL (main score p=0.041). In addition (ii), high cytoplasmatic expression of TNF-Receptor-1 and a strong cytoplasmatic and nuclear expression of CD95 correlated significantly with a better prognosis of the PDAC patients.

Key words: Death ligands, TNFR-SF, Correlations, Immunohistochemistry, PDAC

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Introduction

Apoptosis is a paradigmatic form of programmed cell death, which can be initiated by a great variety of extrinsic and intrinsic signals. The canonical signalling results in DNA-fragmentation, degradation of the cell skeleton and nuclear proteins and finally phagocytosis of the cell (Elmore, 2007) and plays a major role in cancer (Hassan et al., 2014). Activation of the surface receptors CD95, TNF-Receptor and TRAIL-Receptor may also lead to the activation of protein kinase C and NFkB (Trauzold et al., 2001; Roder et al., 2011), resulting in non-apoptotic, pro-inflammatory signaling.

Pancreatic ductal adenocarcinoma (PDAC) is characterized by a poor prognosis and limited therapeutic options. One mechanism, by which PDAC cells escape non-surgical therapy is the activity of multiple apoptosis resistance mechanisms. Although death receptor- and corresponding ligand-expression in PDAC cells have been investigated in previous studies (Arlt et al., 2013) little is known about the co-expression of the different death receptors and its correlation with

patient survival. Various studies explored TRAIL-Receptors, as putative targets for anti-cancer treatment (Lim et al., 2015; de Miguel et al., 2016). However, many tumor cells are resistant against TRAIL-induced apoptosis, and in these cells TRAIL can even promote malignancy and tumor growth (Trauzold et al., 2006). Within the human TRAIL-Receptor family, only TRAIL-Receptor-1 and -2 are able to induce apoptosis. The other TRAIL-Receptors, i.e. TRAIL-Receptor-3 and -4, lack an active death domain and may act as decoy receptors, which may inhibit apoptosis by the competitive binding of TRAIL. Therefore, the effect of TRAIL is partly dependent on the receptor expression at the cell surface. Apart from TRAIL-Receptor expression at the cell surface, nuclear expression may also exert biological effects on cell survival and cell death (Trauzold et al., 2006). In comparison to non-neoplastic duct cells, PDAC-cells expressed higher levels of nuclear TRAIL-Receptor-2 and, moreover, this expression correlated significantly with a worse survival of patients (Haselmann et al., 2014). TRAIL-Receptor-1 and -2 at the plasma membrane may also lead to cell death via necroptotic signalling. Necroptosis is described as another form of regulated cell death via death receptors and is believed to be a more potent inducer of inflammation than apoptosis (Philipp et al., 2016). A crucial role for the biological effects of TRAIL is determined by the non-canonical signalling of TRAIL-Receptors resulting in activation of NFκB, since this signalling pathway affects a multitude of apoptosis-modulating target genes, for example by blocking apoptosis-relevant pathways (Wang et al., 1998; Wajant, 2017). Yet, the role of intracellular TRAIL-Receptors is poorly understood. As the presence of receptors in the trans-Golgi network or in a soluble form within the cytosol is suspected to serve as a hide-out-mechanism for apoptosis resistance, nuclear TRAIL-receptors were found to inhibit the processing of pri-let-7 miRNA and therefore promoting cell malignancy and metastasis (Bertsch et al., 2014).

TNFR1-TNF-interaction in pancreatic tumor cells is double-edged. A loss of the receptor leads to suppression of immunosurveillance mechanisms and stimulation of angiogenesis via tumor-infiltrating CD4+ T-cells (Muller-Hermelink et al., 2008). However, stimulation of TNFR1 with exogenous TNF results in enhanced tumor progression and metastasis via non-apoptotic signalling (Chopra et al., 2013) resulting in a therapeutic option to intervene with clinically well-established inhibitors of TNF signalling also in pancreatic cancer (Egberts et al., 2008).

CD95 activation may lead to tumor cell cycle stimulation and induces tumor cell migration (Teodorczyk et al., 2015). CD95L can induce non-apoptotic pathways, such as NFκB signalling, the MAP-Kinase-cascades and PKC (Siegmund et al., 2007). An increased serum level of soluble CD95L has been associated with progression of pancreatic carcinomas (Peter et al., 2015). The adaptor molecule of CD95, TRAF2, has been described as overexpressed in PDAC and is able to block apoptosis via activation of NFκB and stimulates cell invasiveness via IL-8, uPA and MMPs (Trauzold et al., 2005). Furthermore, CD95 activation induces cell migration via the PI3K/AKT/GSK3β pathway in glioblastomas (Kleber et al., 2008).

In summary, TRAIL, TNF and CD95L are capable of promoting cell death due to caspase-driven apoptosis or caspase-independent necroptosis and they are also able to initiate anti-apoptotic pathways, leading to an increased cell survival and advanced malignancy via stimulating ERK1/2, JNK or NFκB, which results in inflammation and tumor progression. The biological effect of death receptors is determined by the cellular and tissue context, which regulates the balance between apoptotic and non-apoptotic signalling (Roder et al., 2011). It has been further shown that apoptosis resistance mechanisms in PDAC-cells are either constitutively expressed or activated after treatment with chemotherapeutics, e.g. via the induction of NFκB through the activation of PKC (Arlt et al., 2003, Trauzold et al., 2001). NFκB is not only a common mediator of signals induced by the three members of the death receptor (DR)-family but also a master switch in inducing the expression of these receptors (Oeckinghaus et al., 2011; DiDonato et al., 2012). This interplay and the mutual interaction on the level of regulating the expression of members of the TNFR-SF as well as their functional crosstalk has not been fully understood so far. Thus, the possible interrelation of the TNF receptor superfamily members on the tumor tissue level warrants further investigation.

Materials and methods

Study population

From the archive of the Department of Pathology, University Hospital Schleswig- Holstein (UKSH) and Christian-Albrechts-University Kiel, we identified patients who had undergone pancreatectomy for ductal adenocarcinomas of the pancreas between 1999 and 2009. The following patient characteristics were retrieved: type of surgery, age at diagnosis, gender, tumor size, tumor type, tumor grade, depth of invasion, number of lymph nodes resected, and number of lymph nodes with metastases. Inclusion criteria were defined as follows: 1 histology confirmed a ductal adenocarcinoma of the pancreas; 2 tumor stage was pT3N1M0, according to the UICC "TNM Classification of Malignant Tumors", 7th edition. Patients were excluded when histology identified a tumor type other than adenocarcinoma or when histopathological data were incomplete.

Finally 41 patients with diagnosis of stage pT3N1M0 were selected (Table 1).

Clinical data were obtained from the Biomaterial Bank BMB-CCC, UKSH, Kiel. Date and cause of the

patient's death were obtained from the Epidemiological Cancer Registry of the State of Schleswig-Holstein, Germany. The study was approved by the local ethics committee of the Medical Faculty of the Christian-Albrechts-University Kiel, Germany (reference number A 110/99).

Histology and TNM classification

Tissue specimens obtained from routine surgical resection specimens were fixed in formalin and embedded in paraffin. Deparaffinized sections were stained with hematoxylin and eosin. Tumors were classified according to the WHO-criteria. pTNM-stage of all study patients was determined according to the AJCC Cancer Staging Manual, 7th Edition (2010) (Edge and Compton, 2010) and was based solely on surgical pathological examination by board certified surgical pathologists.

Immunohistochemistry

For immunostaining, 2 μm thin paraffin-tissue sections were cut and placed onto SuperFrost plus glass slides (Menzel-Gläser, Braunschweig, Germany). Staining was done manually. After deparaffinization (2 changes of xylene for 10 minutes each; 2 changes of 96% ethanol for 2 minutes each; 2 changes of 70% ethanol for 2 minutes each; 2 changes of 50% ethanol for 2 minutes each; 3 changes of deionized H_2O for 2 minutes each) antigen retrieval was done, using EDTA (pH 8,0), Citrate (pH 6,0) or Tris-HCL (pH 7,0) buffer for 5 to 20 min at boiling temperature in a pressure cooker (Table 2). After buffering (TBS 1x5 min), Peroxidase Block (incubation at room temperature for 15 min), followed by another buffering (TBS 3x5 min) and UV-Block (incubation at room temperature for 5 min) (Thermo Scientific, Waltham, USA) preceded the primary antibodies, which were diluted in Antibody Diluent (Zytomed Systems, Berlin, Germany). Incubation with antibody diluent only served as negative control. Incubation was performed overnight at 4°C. Washing was done with TBS for 3x5 min, then Peroxidase-Polymer (Histofine, Nichirei Biosciences, Tokyo) was used as secondary antibody reagent (incubation at room temperature for 30 minutes). After

Table 1. Clinical and demographic patient data.

ID	sex	Age at surgery	Age at death	Survival [months]
1	f	63	71	101
2	m	65	68	34
3	m	65	67	29
4	f	74	74	5
5	m	69	69	4
6	m	65	65	1
7	m	63	67	43
8	m	47	47	8
9	f	75	75	7
10	f	70	70	1
11	f	61	61	5
12	m	62	62	5
13	f	77	77	6
14	m	63	66	39
15	f	78	79	12
16	f	81	82	1
17	m	54	63	108
18	f	76	77	9
19	m	54	57	43
20	m	81	82	11
21	f	67	69	30
22	f	58	62	51
23	m	75	75	1
24	m	75	77	23
25	m	64	66	21
26	m	71	74	32
27	f	55	59	55
28	f	66	70	43
29	m	51	52	12
30	m	68	68	5
31	m	54	56	37
32	f	49	50	16
33	m	50	50	5
34	f	63	66	38
35	f	76	76	2
36	m	60	60	1
37	f	85	86	15
38	m	77	77	4
39	f	61	62	12
40	m	74	74	0
41	m	66	66	0

Table 2. Staining conditions for antibodies investigated.

Antibody	Source	Antibody characteristics	Pretreatment	Antibody dilution	Chromogen
Anti-TRAIL-R 1.02	Prof. H. Walczak, London	Mouse, monoclonal	EDTA *	1:200	DAB 6 min
Anti-TRAIL-R 2.21	Prof. H. Walczak, London	Mouse, monoclonal	Citrate **	1:150	DAB 6 min
Anti-TRAIL-R 4.18	Prof. H. Walczak, London	Mouse, monoclonal	EDTA *	1:150	DAB 6 min
Anti-TRAIL-Ligand (sab3500424)	Sigma Aldrich, St. Louis, USA	Rabbit, polyclonal	Citrate **	1:1000	DAB 10 min
Anti-CD95-R (ABIN97060)	Antibodies Online, Aachen, Germany	Rabbit, polyclonal	Citrate **	1:350 ***	DAB 6 min
Anti-TNF-R1 (ab19139)	Abcam, Cambridge, England	Rabbit, polyclonal	Citrate **	1:400 ***	DAB 6 min
Anti-TNF-Ligand (ab6671)	Abcam, Cambridge, England	Rabbit, polyclonal	Citrate **	1:300 ***	DAB 6 min

*: antigen retrieval procedure: heat the buffer to boiling temperature, switch off heat and incubate for 10 min, reheat buffer to boiling temperature and incubate for another 5 min. **: antigen retrieval procedure: heat the buffer to boiling temperature, incubate for 5 min. ***: before storing at 4°C overnight, the sections incubated for 30 min at room temperature after applicating the primary antibody.

rinsing (TBS 3x5 min), diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA, USA) served as chromogen. Washing was performed using deionized water for 5 min. Sections were counterstained with Mayer's hemalum solution (Holborn und Söhne, Leipzig, Germany). Dehydration of the sections was done (deionized H_2O for 5 minutes; 2 changes of 50% ethanol for 1 minute each; 2 changes of 70% ethanol for 1 minute each; 2 changes of 96% ethanol for 1 minute each; 2 changes of xylene for 1 minute each) before the slides were covered with mounting medium (Medite GMBH, Burgdorf) and coverslips (Menzel-Gläser, Braunschweig).

Evaluation

Immunoreactivity of the examined receptors and ligands were evaluated separately for the nucleus and the cytoplasm of the tumor cells. The percentage of immunostained cells was categorized into "absent" (0% staining); "sparse" (<10% cells stained); "moderate" (10% to 50%); "substantial" (51% to 90%); or "nearly complete" (>90%). The intensity of immunoreaction was categorized as "negative", "weak", "moderate" or

Table 3. Histopathological analysis, semiquantitative results.

	TRAIL-R-1			TRAIL-R-2			TRAIL-R-4			TRAIL			CD95-R			TNF-R1			TNF		
Patient	C	N	M	C	N	M	C	N	M	C	N	M	C	N	M	C	N	M	C	N	M
1	1	2	3	2	3	5	1	0	1	2	0	2	2	2	4	2	0	2	0	0	0
2	X	X	X	X	X	x	1	0	1	2	0	2	2	3	5	2	0	2	1	0	1
3	X	X	X	X	X	X	X	X	X	2	0	2	2	0	2	X	X	X	2	0	2
4	X	X	x	2	0	2	1	0	1	2	0	2	2	0	2	0	0	0	0	0	0
5	X	X	X	X	X	X	1	0	1	2	0	2	2	0	2	2	0	2	X	X	X
6	1	3	4	2	1	3	0	0	0	2	3	5	2	2	4	2	0	2	1	0	1
7	0	0	0	1	0	1	1	0	1	1	2	3	2	3	5	1	2	3	0	0	0
8	1	1	2	2	2	4	0	0	0	1	0	1	2	2	4	1	0	1	0	0	0
9	X	X	X	X	X	X	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0
10	1	2	3	2	3	5	0	0	0	1	0	1	2	0	2	1	0	1	1	0	1
11	2	3	5	X	X	X	1	1	2	1	1	2	2	1	3	2	1	3	0	0	0
12	1	1	2	1	2	3	1	0	1	1	0	1	2	0	2	1	0	1	1	0	1
13	2	3	5	X	X	X	2	0	2	2	3	5	2	1	3	2	0	2	2	0	2
14	2	3	5	1	2	3	1	1	2	1	0	1	2	1	3	2	0	2	0	0	0
15	2	3	5	2	3	5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
16	0	0	0	1	0	1	1	0	1	1	0	1	1	0	1	1	0	1	0	0	0
17	X	X	X	X	X	X	0	0	0	1	0	1	2	1	3	2	0	2	1	0	1
18	0	0	0	1	3	4	0	0	0	1	0	1	2	0	2	1	0	1	0	0	0
19	0	3	3	1	3	4	1	0	1	2	3	5	2	1	3	2	1	3	1	0	1
20	2	3	5	2	2	4	1	0	1	2	0	2	2	3	5	2	0	2	0	0	0
21	2	3	5	2	3	5	1	0	1	0	0	0	2	0	2	2	0	2	1	0	1
22	1	1	2	2	3	5	2	0	2	1	0	1	2	2	4	1	0	1	0	0	0
23	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
24	0	2	2	2	3	5	1	0	1	2	2	4	2	1	3	2	0	2	1	0	1
25	X	X	X	X	X	X	1	0	1	2	0	2	2	3	5	2	0	2	0	0	0
26	2	3	5	2	3	5	1	0	1	2	2	4	2	0	2	1	0	1	0	0	0
27	1	1	2	2	1	3	1	0	1	2	0	2	2	3	5	2	0	2	1	0	1
28	2	3	5	2	0	2	1	0	1	2	0	2	2	3	5	2	0	2	2	0	2
29	X	X	X	X	X	X	2	0	2	2	0	2	2	2	4	2	0	2	1	0	1
30	1	3	4	2	3	5	0	0	0	2	0	2	2	3	5	2	0	2	1	0	1
31	X	X	X	X	X	X	2	0	2	2	1	3	2	1	3	2	0	2	2	0	2
32	2	1	3	2	3	5	0	0	0	1	0	1	2	1	3	1	0	1	X	X	X
33	2	3	5	1	3	4	0	0	0	1	0	1	1	0	1	1	0	1	0	0	0
34	0	0	0	2	3	5	0	0	0	0	0	0	2	0	2	0	0	0	0	0	0
35	X	X	X	X	X	X	1	0	1	2	2	4	1	0	1	2	2	4	0	0	0
36	2	1	3	2	3	5	2	0	2	X	X	X	2	0	2	1	0	1	0	0	0
37	2	3	5	2	3	5	0	0	0	1	0	1	2	2	4	2	1	3	1	0	1
38	2	2	4	2	3	5	1	0	1	1	1	2	1	0	1	2	0	2	1	0	1
39	X	X	X	X	X	X	0	0	0	2	0	2	2	1	3	1	0	1	0	0	0
40	0	3	3	2	3	5	1	0	1	0	0	0	2	0	2	1	0	1	0	0	0
41	1	0	1	0	0	0	X	X	X	1	0	1	1	0	1	0	0	0	0	0	0

C: Score Cytoplasma (0= neg; 1= weak intensity in 10%-100% of PDAC cells or medium intensity in <50% of PDAC cells; 2= medium intensity in <50% of PDAC cells or strong intensity in 10%-100% of PDAC cells). N: Score Nucleus (0= neg; 1= low or medium intensity in 10% of PDAC cells; 2= low intensity in 11-80% in PDAC cells or high intensity in 10% of PDAC cells; 3= medium or high intensity in 11-80% of PDAC cells or any intensity in 100% of PDAC cells). M: Main Score, the result of summing the nuclear and cytoplasmatic score. X: Cases were excluded.

"strong". Details are shown in Table 3.

Statistical analysis

Statistical Analysis was carried out using the PC software IBM SPSS-Statistics version 19 (IBM Corporation). For continuous variables, cases were divided into two groups by splitting at the median value. For the correlation analysis regarding the antigen expression Kendall's Tau rank correlation coefficient was used as a non-parametric statistic mean to measure the association between two measured ordinal variables. As correlation coefficients possess values between -1 and +1, the positive correlation signifies that the ranks of both the variables are increasing, whereas, on the other hand, the negative correlation signifies that as the rank of one variable is increased, the rank of the other variable is decreased. Kaplan-Meier analysis was used for the survival analysis, counting in months from the date of surgery until the patients' death. Patients without known date of death (lost in follow up or alive) were censored. The corresponding p-values were obtained by using the Log-Rank-Test. A P-value <0.05 was considered as statistically significant.

Table 4. Summary of correlating expression data of TNFR-SF members and cognate ligands. See text for further details.

		Subcellular distribution and main score
TNFR1	TNFα	cytoplasmatic, nuclear
TNFR1	TRAIL	nuclear, main score
TNFR1	CD95	main score
TR1	TR2	nuclear
TR4	TRAIL	main score

Results

Patient cohort

Patient data are shown in Table 1. The cohort consisted of 18 women and 23 men. The mean age of death for the entire cohort of patients was 67.7 years (female: 70.3 years, male: 65.6 years). Within the median overall survival of 21.3 months in our small cohort a slightly better prognosis was recorded in female patients with a median survival of 22.7 months whereas in male patients it was 20.2 months. Median overall survival was 21.3 months.

Immunohistochemistry

The TRAIL-Receptors-1, -2, -4, the ligand TRAIL, CD95 and TNFR were all detected in the majority of the pancreatic tumor cells, mostly in both the cytoplasm and the nucleus. An overview of the findings is presented in Table 4.

TRAIL-Receptor-1

TRAIL-Receptor-1 could be assessed in 39 PDACs. 33 (84.6%) cases showed cytoplasmic and 29 (74.4%) nuclear immunostaining (Fig. 1A,B). In 28 cases

Table 5. Correlation of survival and antigen expression.

Staining	TRAIL-R1			TRAIL-R2			TRAIL-R4			TRAIL			CD95			TNF-R1			TNF-alpha		
parameters	n	Survival	p	n	Survival	p	n	Survival	p	n	Survival	p	n	Survival	p	n	Survival	p	n	Survival	p
Cytoplasmatic Intensity																					
neg. & weak	22	8 (0.0-17.1)	0.858	18	9 (0.0-19.4)	0.546	29	12 (1.5-22.5)	0.545	19	8 (2.3-13.7)	0.638	5	4 (0.0-12.6)	0.001	15	5 (0.0-10.7)	0.031	26	9 (2.8-15.2)	0.806
med. & strong	17	12 (6.6-17.4)		20	11 (2.2-19.8)		8	6 (0.0-21.2)		18	12 (0.0-25.9)		33	15 (3.7-26.2)		23	21 (3.8-38.2)		10	12 (0.0-38.3)	
Cytoplasmatic Quantity																					
<80%	14	7 (1.5-12.5)	0.567	4	5 (0.8-9.2)	0.552	25	12 (2.2-21.8)	0.984	14	7 (0.0-14.3)	0.177	23	Average: 2.5 (0.0-7.40)	0.027	20	9 (0.0-20.0)	0.094	35	11 (5.2-16.8)	0.769
>80%	25	12 (5.5-18.5)		34	12 (3.4-20.6)		12	6 (0.0-17.9)		23	12 (0.0-27.7)		6	12 (3.2-20.8)		18	11 (2.7-19.3)		1	37	
Nuclear Intensity																					
neg. & weak	22	7 (2.4-11.6)	0.689	15	12 (0.0-29.7)	0.304	35	12 (3.9-20.1)	0.998	27	12 (7.0-17.1)	0.671	16	4 (1.6-6.4)	0.000	35	11 (5.4-16.6)	0.793	36	11 (5.1-16.9)	-
med. & strong	17	12 (8.0-16.0)		23	9 (0.0-20.0)		2	5±0.0		10	6 (0.0-33.9)		22	21 (0.0-42.8)		5	15 (0.0-36.5)		0	-	
Nuclear Quantity																					
<10%	18	8 (3.8-12.2)	0.741	11	7 (0.0-14.6)	0.619	37	12 (3.7-20.3)	-	32	12 (3.7-20.3)	0.915	26	6 (2.4-9.6)	0.349	38	11 (5.0-17.0)	-	36	11 (5.1-16.9)	-
>10%	21	12 (10.5-13.5)		27	12 (5.2-18.8)		0	-		5	6 (0.0-14.6)		12	15 (0.0-30.3)		0	-		0	-	

Survival times are listed in months. The median survival was obtained by using Kaplan Meier.

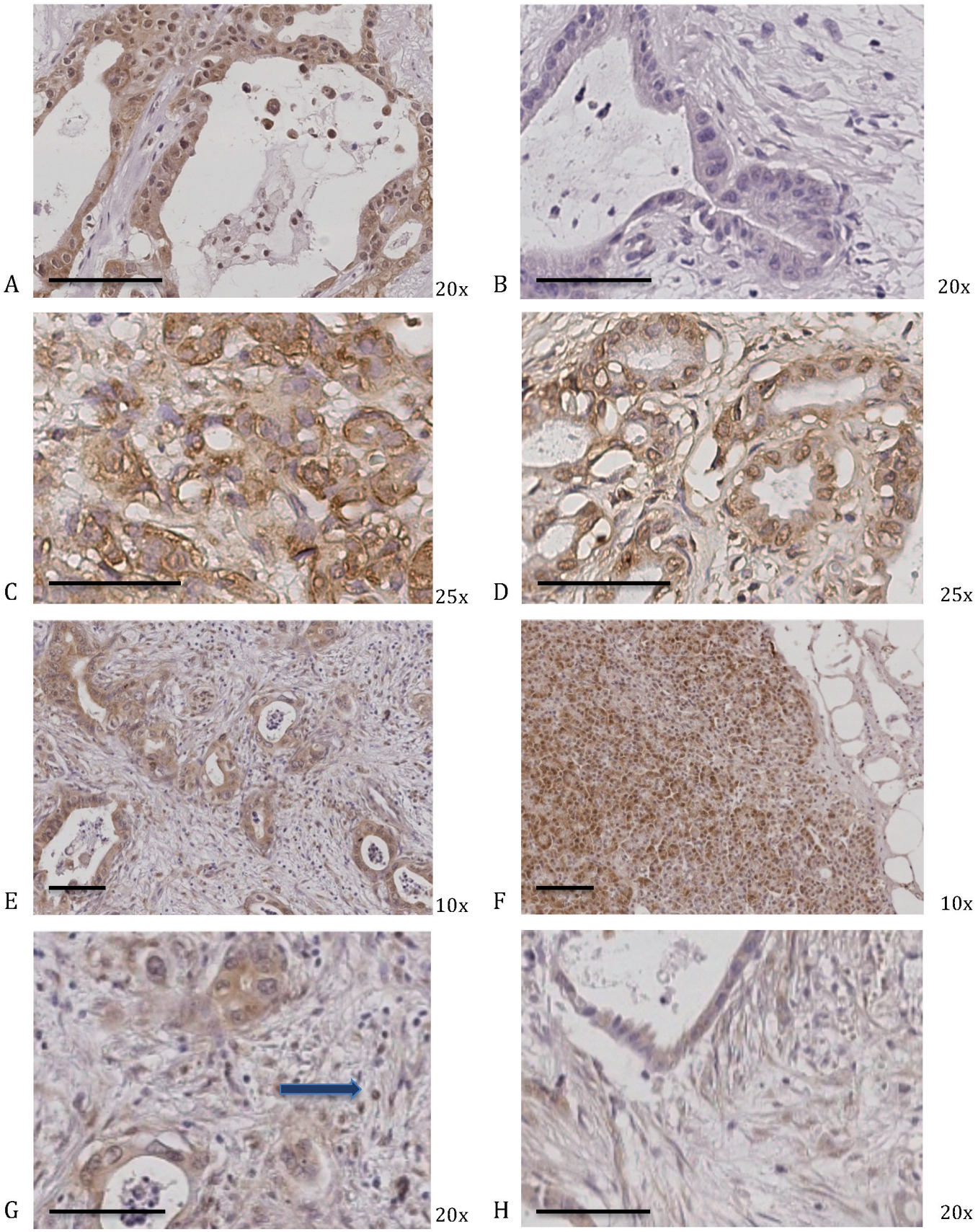

Fig. 1. Representative examples of immunohistological stainings. **A.** TRAIL-R1: strong cytoplasmatic and partial nuclear expression. **B.** TRAIL-R1: Negative/weak expression, no stroma positivity. **C.** TRAIL-R2: strong cytoplasmatic expression. **D.** TRAIL-R2: nuclear expression, in particular at the membrane. **E.** TRAIL-R4: strong cytoplasmatic expression. **F.** TRAIL-R4: immunoreactivity in pancreatic acinar cells. **G.** TRAIL-R4: immunoreactivity in lymphocytes (arrow). **H.** TRAIL: weak immunoreactivity.

(71.8%) more than 50% of the tumor cells expressed TRAIL-Receptor-1 in the cytoplasm, mainly with weak or medium staining intensity. Nuclear immunoreactivity was found in 35.9% in more than half of the tumor cells.

TRAIL-Receptor 2

Except for one case with no staining, cytoplasmatic expression of TRAILR-2 was found in all PDAC cases (97.4%). In 34 cases (89.5%) more than 80% of the tumor cells showed cytoplasmatic immunoreactivity for TRAILR-2 (Fig. 1C). Nuclear staining was observed in 33 of 38 cases (86.8%) (Fig. 1D). TRAILR-2 expression was detected in 16 of 38 cases (42.1%) in more than 80% of the tumor cells.

TRAIL-Receptor 4

Immunoreactivity was detected in lymphocytes (Fig. 1G), erythrocytes (not illustrated), acini (Fig. 1F) and tumor cells. Cytoplasmatic TRAILR-4 in PDAC cells was observed in 25 of 37 cases (67.6%) (Fig. 1E), mostly with weak intensity (45.9%). In two cases (5.4%) weak nuclear immunoreactivity was detected in a few PDAC cells (<10%) (not illustrated).

TRAIL

Cytoplasmatic staining overall was detected in 93.9% of the PDAC cells, in particular in perinuclear regions. In 43.2% cytoplasmatic staining intensity was weak (Fig. 1H). Moreover, pancreatic islet cells (not illustrated), stromal cells, nerve cells and endothelial cells (Fig. 2A) showed TRAIL expression. Nuclear expression was observed in 10 of 37 (27%) cases, in 18.9% in less than 50% of the PDAC cells (not illustrated).

CD95

Cytoplasmatic expression was found in every case, strong intensity was detected in 63.2% (Fig. 2B). 36 of 38 cases (94.7%) showed cytoplasmatic CD95 expression in more than 80% of the PDAC cells. Nuclear antigen expression was found in 22 cases (57.9%) (not illustrated). Besides PDAC cells, immunoreactivity was also observed in granulocytes (Fig. 2C).

TNF-Receptor 1

Cytoplasmatic TNF-R1-expression was detected in 89.5 % (34 of 38) of the PDAC cases. In 47%, more than 80% of the tumor cells were positive (Fig. 2D,E). Nuclear expression of TNF-R1 was found in 5 of 38 cases in less than 10% of the PDAC cells (not illustrated). Moreover, immunoreactivity was detected in pancreatic acinar cells (Fig. 2G) and in granulocytes (Fig. 2F).

TNFα

All cases showed a strong immunoreactivity in stromal cells. Cytoplasmatic staining was found in 44.4% (20 of 36) in the tumor cells of the specimen, mostly in less than 50% of the cells. No nuclear immunoreactivity was observed in the PDAC cells (Fig. 2H).

Correlation of expression of TNFR-SF members and cognate ligands (i)

The respective antigen co-expression in the malignant cells of the PDAC-tissues was investigated according to the respective subcellular distribution. A highly significant correlation was found between TNF-Receptor 1 and its ligand TNFα regarding cytoplasmatic and nuclear scores (e.g. cytoplasmatic score, containing intensity and quantity $\tau=+0.545$, $p=0.001$). The positive τ-correlation signifies that the ranks of both the variables are increasing. The expression of TNF-Receptor 1 and TRAIL correlated as well concerning the quantity of nuclear antigen expression ($\tau=+0.497$, $p=0.002$) and also in terms of the main score, which is the result of the summation of cytoplasmatic and nuclear scores and therefore describes the overall intracellular antigen expression ($\tau=+0.366$, $p=0.016$). Regarding the main score, a highly significant co-expression was also observed between TNF-Receptor-1 and CD95 ($\tau=+0.414$, $p=0.003$). TRAIL-Receptor-1 and TRAIL-Receptor-2 showed a significant correlation in the nuclear parameters (e.g. nuclear score, containing intensity and quantity $\tau=+0.313$, $p=0.023$). Concerning the main score, a co-expression of TRAIL-Receptor-4 and TRAIL was found ($\tau=+0.299$, $p=0.041$). All the significant results of antigen correlations are summarized in Table 5.

Expression of TNFR-SF members and cognate ligands in correlation to survival (ii)

Neither TRAIL-Receptor 1, -2, -4, nor TRAIL expression analysis revealed any significant correlation with the cumulative survival in this cohort. However, a significant correlation was found for TNF-Receptor-1 and CD95 expression with the survival time. A medium or strong cytoplasmatic expression of TNF-Receptor-1 was associated with a longer survival (Fig. 3; median survival 21 months, SE 8.8 months, p=0,031) than a weak or no expression (median survival 5 months, SE 2.9 months, p=0.031). Regarding cytoplasmatic parameters, a medium or strong expression of CD95 correlated significantly with longer survival (e.g. cytoplasma score, including staining intensity and quantity rate: median survival 15 months, SE 5.7 months) versus no or weak expression pattern (median survival 2 months, SE 2.4 months, $p<0.001$). The same tendency was observed in the CD95 nuclear analysis (Fig. 4). A medium or strong nuclear staining intensity

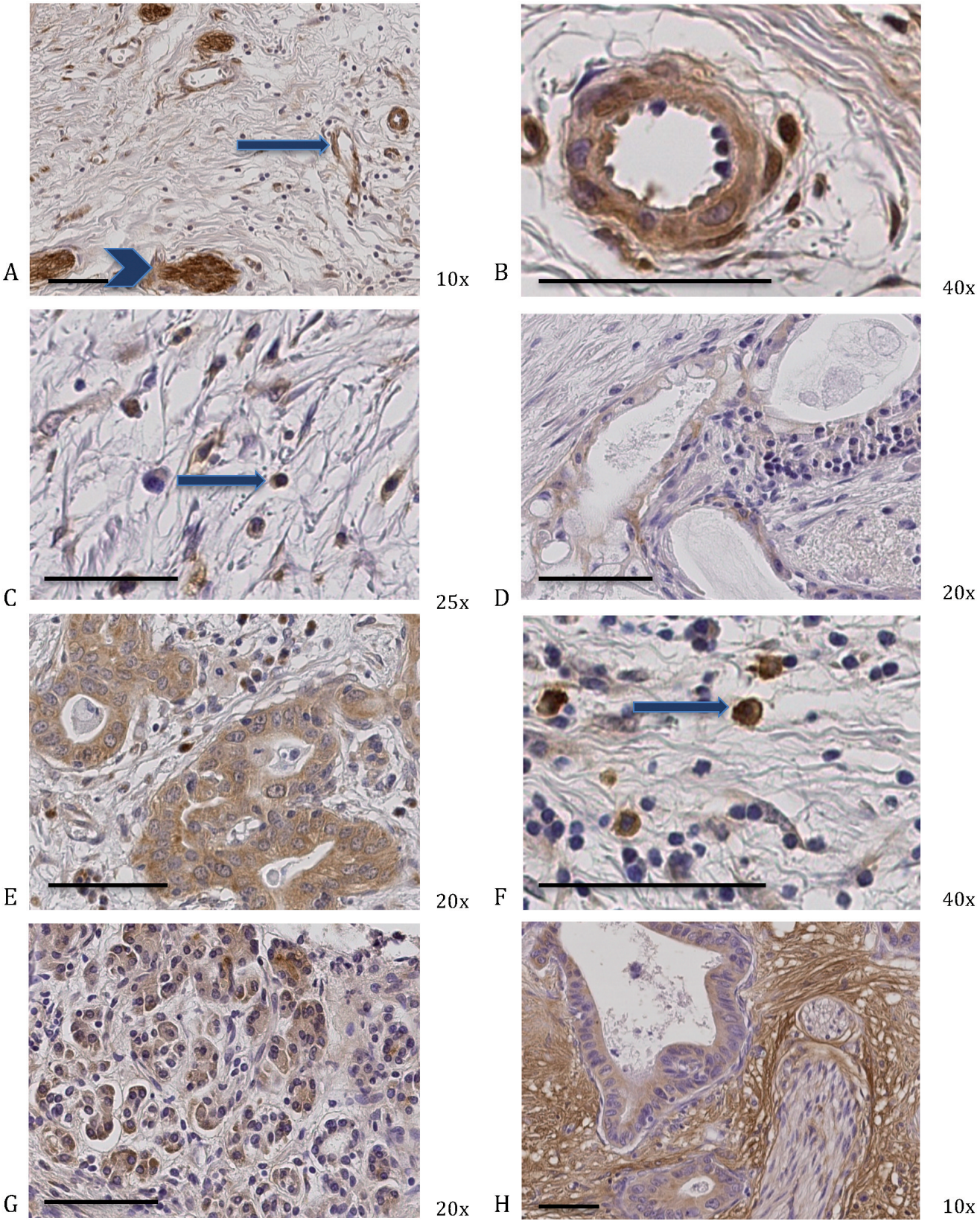

Fig. 2. Representative examples of immunohistological stainings. **A.** TRAIL: strong expression in neural cells (short arrow) and endothelial cells (long arrow). **B.** CD95: strong cytoplasmatic immunoreactivity with sporadic nuclear staining. **C.** CD95: immunoreactivity in granulocytes (arrow). **D.** TNF-R1: weak cytoplasmatic staining. **E.** TNF-R1: strong cytoplasmatic staining. **F.** TNF-R1: immunoreactivity in granulocytes (arrow). **G.** TNF-R1: immunoreactivity in pancreatic acinar cells, cytoplasmatic staining in PDAC cells. **H.** TNF α: cytoplasmatic staining, in particular at the luminal side; no nuclear positivity was detected; strong immunoreactivity in the stroma cells.

was associated with a longer survival (median survival 21 months, SE 11.1 months, p≤0,001) than no or a weak staining intensity (median survival 4 months, SE 1.2 months, p≤0,001). Detailed results are shown in Table 6.

Discussion

Pancreatic adenocarcinoma has a very poor prognosis and is characterized by an early metastatic spread and high resistance towards many different therapies (Raimondi et al., 2009). This finding is consistent with a large retrospective review of 955 PDAC-patients. The median survival of patients with T3-category was 24.0 months and for any N+-category it was 21.9 months (Morganti et al., 2014). Another analysis of 91 PDAC-patients with the UICC stage II showed a median survival time of 22 months (Dhayat et al., 2015).

Apoptosis induction by external triggers like death ligands have been largely shown to be insufficient, although many pancreatic cancer cells express death receptors (Roder et al., 2011). Although these receptors have been investigated in the past, little is known about death receptor/ligand co-expression in PDAC-cells.

We investigated the expression of TRAIL-Receptors-1, -2, -4; TNF-Receptor-1, CD95 and their ligands TRAIL and TNFα in the nucleus and cytoplasm of pancreatic cancer cells in clinical PDAC specimens.

Table 6. Patterns of antigen expression intensity in the PDAC tissue samples and the proportion (%) of positive cells.

Staining	TRAIL-R1		TRAIL-R2		TRAIL-R4		TRAIL		CD95		TNF-R1		TNF-alpha	
parameters	Cyto. [%]	Nucl. [%]	Cyto. [%]	Nucl. [%]	Cyto. [%]	Nucl. [%]	Cyto. [%]	Nucl. [%]	Cyto. [%]	Nucl. [%]	Cyto. [%]	Nucl. [%]	Cyto. [%]	Nucl. [%]
Intensity														
negative	6 (15,3)	10 (25,6)	1 (2,6)	5 (13,2)	12 (32,4)	35 (94,6)	3 (8,1)	27 (73,0)	0 (0,0)	16 (42,1)	4 (10,5)	33 (86,8)	20 (55,6)	36 (100)
weak	16 (41,0)	12 (30,8)	17 (44,7)	10 (26,3)	17 (45,9)	2 (5,4)	16 (43,2)	4 (10,8)	5 (13,2)	3 (7,9)	11 (28,9)	1 (2,6)	6 (16,7)	0 (0,0)
medium	10 (25,6)	9 (23,0)	12 (31,6)	10 (26,3)	2 (5,4)	0 (0,0)	9 (24,3)	3 (8,1)	9 (23,7)	15 (39,5)	8 (21,1)	1 (2,6)	10 (27,8)	0 (0,0)
strong	7 (17,9)	8 (25,1)	8 (21,1)	13 (34,2)	6 (16,2)	0 (0,0)	9 (24,3)	3 (8,1)	24 (63,2)	4 (10,5)	15 (39,5)	3 (7,9)	0 (0,0)	0 (0,0)
Quantity: N (%)														
0%	6 (15,3)	10 (25,6)	1 (2,6)	5 (13,2)	12 (32,4)	35 (94,6)	3 (8,1)	27 (73,0)	0 (0,0)	16 (42,1)	4 (10,5)	33 (86,8)	20 (55,6)	36 (100)
<10%	3 (7,7)	8 (25,1)	1 (2,6)	6 (15,8)	2 (5,4)	2 (5,4)	5 (13,5)	5 (13,5)	0 (0,0)	10 (26,3)	5 (13,2)	5 (13,2)	5 (13,9)	0 (0,0)
11-50%	2 (5,1)	7 (17,9)	0 (0,0)	5 (13,2)	7 (18,9)	0 (0,0)	2 (5,4)	2 (5,4)	1 (2,6)	7 (18,4)	2 (5,3)	0 (0,0)	7 (19,4)	0 (0,0)
51-80%	3 (7,7)	2 (5,1)	2 (5,3)	6 (15,8)	4 (10,8)	0 (0,0)	4 (10,8)	3 (8,1)	1 (2,6)	5 (13,2)	9 (23,7)	0 (0,0)	3 (8,3)	0 (0,0)
>80%	25 (64,1)	12 (30,8)	34 (89,5)	16 (42,1)	12 (32,4)	0 (0,0)	23 (62,1)	0 (0,0)	36 (94,7)	0 (0,0)	18 (47,4)	0 (0,0)	1 (2,8)	0 (0,0)

Cyto.: cytoplasmatic, Nucl.: nuclear staining.

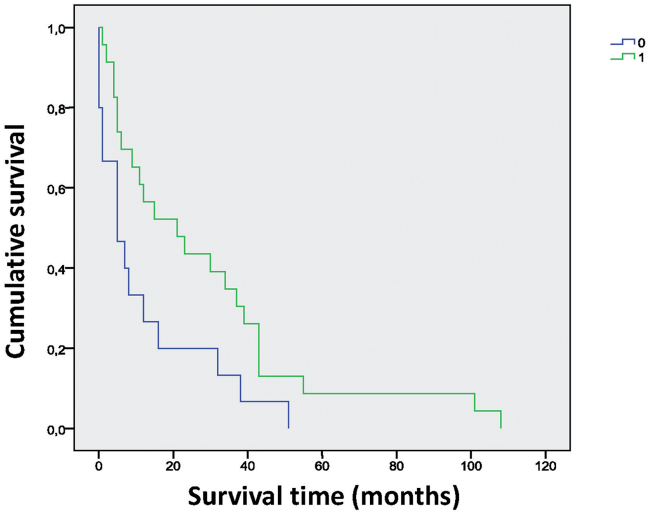

Fig. 3. Kaplan-Meier-analysis of cumulative survival rate in correlation to cytoplasmatic TNFR-expression. Significance (p-value) were calculated using the log-rank-test and significance was considered if p-values were <0.05. Results were dichotomized: 0=no and weak positivity (n=15), 1=medium and strong positivity (n=23).

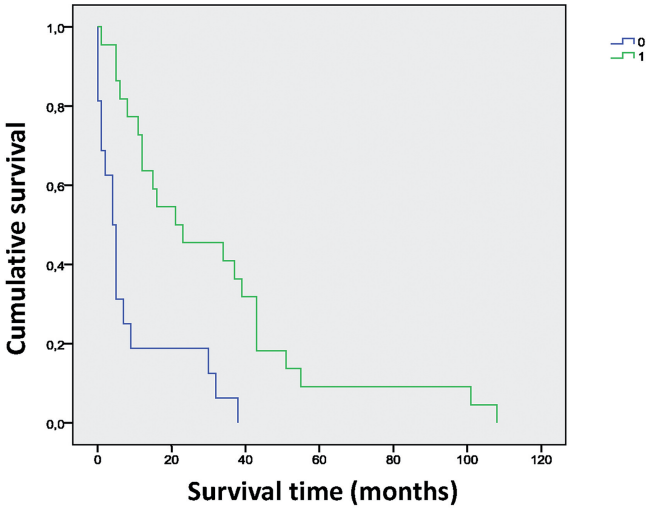

Fig. 4. Kaplan-Meier-analysis of cumulative survival rate in correlation to nuclear CD95 expression. Significance (p-value) were calculated using log-rank-test and significance was considered if p-values were <0.05. Results were dichotomized: 0=no and weak positivity (n=16), 1=medium and strong positivity (n=22).

Many previous publications have studied members of the TNF receptor superfamily in a variety of tumors, yet few attempts have been undertaken comparing the expression of the different receptors in one cohort.

In this study, the TRAIL-Receptors and their ligand were detected in the majority of PDAC cells in nearly all tested specimens. No significant correlation between expression and patient survival was found in this cohort of 41 cases. However, in a previous analysis of a larger and more diverse cohort an upregulation of TRAIL-R2 in PDAC cells was described, which significantly correlated with shorter survival rates (Haselmann et al., 2014). Since survival times for PDAC patients are in general limited, differences in cohort size – like here about threefold – may gain particular impact influencing the final outcome. CD95 was found in the cytoplasm of every case investigated and a nuclear expression was detected in 58% of the cases. A strong expression in the nucleus and cytoplasm correlated significantly with longer survival times. Similarly, a high cytoplasmatic expression of TNF-Receptor-1 was significantly associated with a longer survival. TNF-Receptor-1 was detected in the cytoplasm of the majority of the cases, but nuclear expression was seen rarely. TNFα was only detected in a few cases' cytoplasm and no significant correlation with the patients' survival was found.

Several significant correlations were found in the co-expression studies. TNF-Receptor-1 and its ligand were co-expressed in the cytoplasm and nucleus of the PDAC-cells. This finding is consistent with observations in other tumor types. For example, an upregulation of TNFα and TNF-Receptor-1 is described in the progression of Barret's metaplasia to esophageal adenocarcinoma (Tselepis et al., 2002). Regarding nuclear parameters, TNF-Receptor-1 and TRAIL correlated significantly. A significant co-expression of TRAIL-Receptor-1 and TRAIL-Receptor-2 was found in the nucleus. Regarding the cumulative antigen expression, containing nuclear and cytoplasmatic parameters, TRAIL-Receptor 4 and TRAIL correlated significantly. This might be interesting in the context of recent findings on the role of the endogenous ligand in *KRAS*-mutated cancers like pancreas and lung (von Karstedt et al., 2015).

One weakness of our study may be seen in the lack of direct co-expression analyses with advanced multi-parameter stainings using the identical tissue section. Yet, the intra-tumoral heterogeneity may counteract this and the analysis of multiple sections may thus be advantageous.

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