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Tumour-mediated TRAIL-Receptor expression indicates effective apoptotic depletion of infiltrating CD8+ immune cells in clinical colorectal cancer

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ABSTRACT

Expression of apoptosis-related proteins on tumour cells has been shown in several experimental models to be an efficient mechanism for a counterattack against host anti-tumour immune responses in solid tumours. Here we provide a clinical evidence for such a tumour immune escape mechanism by demonstrating tumour to T cell-directed death receptor signalling (TRAIL/TRAIL-Receptor (TRAIL-R)) in colorectal cancer (CRC). In a series of patients with CRC and completed 5-year follow up, we investigated apoptosis and expression levels of apoptosis-related proteins. Gene and protein profiles in the tumours demonstrated intratumoural upregulated gene expression for Fas, Fas-L, TRAIL, TRAIL-R and TNF-α (RT-qPCR). Levels of terminaldeoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick-end labelling (TUNEL)-positive events were positively correlated with TRAIL-R1-expression on tumour infiltrating immune cells. Among the immune cells, preferentially CD8+ T cells were found to express TRAIL-R1 while serial immunostaining in the same patient tumours showed abundant apoptotic (TUNEL-positive) immune cells. In conclusion, our results in tumour samples from CRC patients suggest TRAIL-R1-mediated apoptotic depletion of infiltrating immune cells (CD8+) in response to TRAIL expression by the tumour itself. This supports the notion of an efficient escape from tumour immune response and thus evasion from the attack of activated CD8+ T cells. These findings may enhance our understanding of tumour progression in CRC and might be helpful for the development of TRAIL and its death receptor-based therapy.

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1. Introduction

Colorectal cancer (CRC) is the third most common cause of cancer-related death worldwide. Between 40% and 50% of the patients die of the disease within 5 years after diagnosis although great advances have been made in the diagnosis and treatment including complete R0-resection of the primary tumour.[1] While many studies recently improved our understanding of the tumour initiation and progression, the phenomenon of an increasingly ineffective tumour immune response during tumour growth still remains unanswered. In fact, during cancer progression, tumour cells acquire various characteristics which allow them to evade immunological surveillance.

Cytokine-induced apoptosis is obviously one of these crucial tumour immune escape mechanisms. Cytokines are messengers of the immune system that can also be released from tumour cells. They are functioning in an autocrine and/or paracrine manner, either locally or at a distance to enhance or suppress immunity. Tumour necrosis factor (TNF), TNF-α is produced by macrophages as well as tumour cells and has multiple effects on cell function by binding to specific, high-affinity cell surface receptors. Beside apoptotic mechanisms, TNF-α may even promote tumour growth at lower levels during cancer progression. Interestingly, during cancer progression tumour cells from CRC have been described to lose their susceptibility for the induction of apoptosis.[2] TNF-related apoptosis-inducing ligand (TRAIL) and Fas-Ligand (Fas-L) are cytokine ligands of the TNF family interacting with different receptors of the TNF receptor superfamily, which can induce apoptosis.[3] The recent concept of TRAIL-mediated apoptosis has attracted wide interest as a potential anticancer therapy with recombinant soluble forms of TRAIL agonists that have been shown to induce cell death predominantly in transformed tumour cells. Several phase II trials have recently been reported to induce partial response or stable disease in a number of patients with solid tumours. The other family members like TNF-α and Fas-L could not advance to the clinic due to their toxic nature.[4]

The strategy of apoptosis in tumour cells clearly represents a concept that should be pursued in tumour patients. Nevertheless, an upregulated process of apoptosis in effector lymphocytes infiltrating the tumour may be a reason for an increasingly ineffective tumour immune response and thus the progression of CRC. Interaction between the tumour cells and the surrounding tumour infiltrating lymphocytes such as Interferon (IFN)-γ producing CD8+ effector T cells and CD56+ natural killer (NK) cells at the tumour border has been shown to play an important role in the malignant potency of breast, gastric and colorectal tumours.[5–7] Upregulated TRAIL expression on tumour cells in CRC may thus help the tumour to defeat infiltrating TRAIL-R1-positive immune effector cells. Therefore, we focussed on TRAIL-R1 that particularly can mediate the apoptotic depletion of the tumour infiltrating cytotoxic T lymphocytes in response to TRAIL expression by the tumour itself.

Many different tumours have been found to express Fas-L suggesting a Fas-mediated counterattack against anti-tumour immune effector cells that may contribute to apoptosis in Fas-positive T-cells.[8] In addition to this event, tumour cells could also be saved from their own weapon because of their resistance to Fas-induced cell death.[9] Furthermore, TRAIL/TRAIL-R1 signalling has been supported to selectively induce cell death in tumour cells.[10,11] To further prove this hypothesis we analysed TRAIL and its death receptor TRAIL-R1 expression as this process may be additionally involved in immunological surveillance.[5–7]

2. Materials and methods

2.1. Patients

Sixty-five patients with histologically proven colorectal cancer undergoing curative surgical resection in our Department between 01/2001 and 06/2004 were included in the study. The histological stage of the tumour was determined according to the Union International Contre le Cancer (UICC)-TNM staging system.[12,13] Tumours were evaluated for location, stage and differentiation grade. Data concerning age, gender, level of wall infiltration and lymph node metastasis were collected in a database, and regular medical visits of the patients after curative therapy were performed at intervals according to the governmental guidelines for tumour patients. None of the patients had undergone neoadjuvant treatment, with any type of preoperative antineoplastic protocol (neither chemotherapy nor radiochemotherapy). Patients with rectal cancer (stages T3 NX and/or TX N+ and higher) having undergone neoadjuvant treatment were excluded from analysis. Only patients who had R0-resections were included in our study. Tumour tissue samples as well as normal colon tissues from the patients were collected with informed consent before surgical resection, frozen instantly in liquid nitrogen, and stored at −80 °C until analysed. With respect to the fact that a colorectal cancer is a complex composition of an in-part necrotising neoplasia interacting with resident stromal components and cells and infiltrating inflammatory cells, we used comparable areas of solid growth distant from the gut lumen. Normal colon tissues from healthy individuals ($n = 12$) as well as patients with colitis (data not shown) served as controls. Clinical characteristics of the study population are summarised in Table 1.

2.2. Quantitative real-time polymerase chain reaction (RT-qPCR)

To analyse gene expression of TRAIL-R1, TRAIL, IFN-γ, Fas-L, Fas, CD8, CD56 and TNF-α by RT-qPCR, we extracted total cellular RNA from the border of the tumour using RNeasy Minikit from Qiagen (Hilden, Germany). RNA extraction was performed according to the manufacturer's instruction. Primers were designed using the Primer Express software for primer design to amplify short segments of 50–150 base pairs of target cDNA or primer sets from Qiagen (Hilden) and R&D Systems (Wiesbaden-Nordenstadt, Germany) were used. Optimum primer concentration was determined by titration. Human colon matched cDNA (*normal colonic mucosa*) was purchased from Pharmingen (Heidelberg, Germany) and used as control. The housekeeping genes glyceraldehyde-3-phos-

Table 1 – Clinicopathological characteristics of the patients.

Characteristics	Patients ($n = 65$)	AI (infiltrating immune cells)		CD8		TRAIL-R1 (infiltrating immune cells)		TRAIL Infiltrating immune cells)		TRAIL-R1 (tumour)		TRAIL (tumour)	
		Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
Age (y)													
⩽64	45 (69%)	22 (49%)	2 (51%)	18 (40%)	27 (60%)	27 (60%)	18 (40%)	20 (44%)	25 (56%)	12 (27%)	23 (73%)	19 (42%)	26 (58%)
⩾65	20 (31%)	9 (45%)	11 (55%)	9 (45%)	11 (55%)	10 (50%)	10 (50%)	9 (45%)	11 (55%)	9 (45%)	11 (55%)	9 (45%)	11 (55%)
Gender													
Male	37 (57%)	18 (49%)	19 (51%)	21 (57%)	16 (43%)	22 (59%)	15 (41%)	12 (32%)	15 (68%)	23 (62%)	14 (38%)	19 (51%)	18 (49%)
Female	28 (43%)	13 (46%)	15 (54%)	6 (21%)	22 (79%)	15 (54%)	13 (46%)	7 (25%)	21 (75%)	8 (29%)	20 (71%)	9 (32%)	19 (68%)
Primary tumour													
Colon	26 (40%)	13 (50%)	13 (50%)	10 (38%)	16 (62%)	20 (77%)	6 (23%)	12 (46%)	14 (54%)	12 (46%)	14 (46%)	12 (46%)	14 (54%)
Rectum	39 (60%)	18 (46%)	21 (54%)	17 (44%)	22 (56%)	17 (44%)	22 (56%)	17 (44%)	22 (56%)	19 (49%)	20 (51%)	16 (41%)	23 (59%)
Histological classification													
G1	12 (18%)	9 (75%)	3 (15%)	3 (83%)	9 (75%)	10 (83%)	2 (17%)	3 (15%)	9 (75%)	3 (15%)	9 (75%)	8 (66%)	4 (34%)
G2	31 (48%)	17 (55%)	14 (45%)	10 (32%)	21 (68%)	19 (61%)	12 (39%)	10 (32%)	21 (68%)	11 (35%)	20 (65%)	17 (55%)	14 (45%)
G3/4	22 (34%)	5 (22%)	17 (78%)	14 (64%)	8 (36%)	8 (36%)	14 (64%)	16 (73%)	6 (27%)	17 (78%)	5 (22%)	3 (14%)	19 (86%)
Depth of invasion													
pT1	14 (22%)	10 (71%)	4 (29%)	4 (29%)	10 (71%)	10 (71%)	4 (29%)	4 (29%)	10 (71%)	3 (21%)	11 (79%)	8 (57%)	6 (43%)
pT2	23 (35%)	15 (65%)	8 (35%)	3 (13%)	20 (87%)	16 (70%)	7 (30%)	3 (13%)	20 (87%)	7 (30%)	16 (70%)	13 (56%)	10 (44%)
pT3	17 (26%)	5 (29%)	12 (71%)	11 (65%)	6 (35%)	6 (35%)	11 (65%)	12 (71%)	5 (29%)	12 (71%)	5 (29%)	6 (35%)	11 (65%)
pT4	11 (17%)	1 (9%)	10 (91%)	9 (82%)	2 (18%)	5 (45%)	6 (55%)	10 (91%)	1 (9%)	9 (82%)	2 (18%)	1 (9%)	10 (91%)
Lymph nodes metastasis													
pN0	34 (52%)	27 (79%)	7 (21%)	8 (24%)	26 (76%)	27 (79%)	7 (21%)	8 (24%)	26 (76%)	8 (24%)	26 (76%)	22 (65%)	12 (35%)
pN1-3	31 (48%)	4 (13%)	27 (87%)	19 (61%)	12 (39%)	10 (32%)	21 (68%)	21 (32%)	10 (68%)	23 (74%)	8 (26%)	6 (19%)	25 (81%)
UICC stage													
UICC I	15 (23%)	14 (93%)	1 (7%)	0 (0%)	15 (100%)	12 (80%)	3 (20%)	0 (0%)	15 (20%)	1(7%)	14 (93%)	11 (73%)	4 (27%)
UICC II	19 (29%)	15 (79%)	4 (21%)	2 (11%)	17 (89%)	14 (74%)	5 (26%)	2 (11%)	17 (89%)	2 (11%)	17 (89%)	14 (74%)	5 (26%)
UICC III	22 (34%)	2 (9%)	20 (91%)	17 (77%)	5 (23%)	8 (36%)	14 (64%)	18 (82%)	4 (18%)	19 (86%)	3 (14%)	2 (9%)	20 (91%)
UICC IV	9 (14%)	0 (0%)	9 (100%)	8 (89%)	1 (11%)	3 (33%)	6 (67%)	9 (100%)	0 (0%)	9 (100%)	0 (0%)	1 (11%)	8 (89%)
Mean OS (m)	32 m	48 ($n = 31$)	16 ($n = 34$)	14 ($n = 27$)	44 ($n = 38$)	41 ($n = 37$)	19 ($n = 28$)	14 ($n = 29$)	46 ($n = 36$)	15 ($n = 31$)	47 ($n = 34$)	47 ($n = 28$)	20 ($n = 37$)
Median OS (m)	31 m	51 ($n = 31$)	10 ($n = 34$)	10 ($n = 27$)	48 ($n = 38$)	46 ($n = 37$)	13 ($n = 28$)	10 ($n = 29$)	50 ($n = 36$)	10 ($n = 31$)	49 ($n = 34$)	52 ($n = 28$)	15 ($n = 37$)

AI, apoptotic index; y, years; G, grading; UICC, International Union against Cancer; OS, overall survival; and m, months.

phate dehydrogenase (GAPDH) and ß-actin were used for relative quantification and cDNA quality control. All PCRs were carried out with a DNA Engine Opticon 2 System (MJ Research, Biozym, Oldendorf, Germany). Reverse transcription from RNA to cDNA was carried out by using iScript cDNA Synthesis Kit (BioRad, Hercules, CA). Each PCR was performed in 23 μl volume containing 11.5 μl the LightCycler-DNA Master SYBR Green I mix (Applied Biosystems, Darmstadt, Germany), 10 pmol/μl forward primer, 10 pmol/μl reverse primer, 3 μl template DNA and 7.5 μl RNase free water. Initial denaturation at 95 °C for 15 min was followed by 39 cycles of a denaturation step at 95 °C for 15 s, an annealing step at 57.5 °C for 30 s and an extension step at 72 °C for 30 s. To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis. The quantification data were analysed with the LightCycler analysis software. Reproducibility was confirmed by an independent PCR repeated twice. The average threshold cycle (Ct) value was calculated as the cycle number at which the fluorescence of the reporter reaches a fixed threshold. The difference (ΔCt) between the average Ct values of the samples in the target wells and those of the housekeeping gene, GAPDH or ß-actin, was assessed, followed by the calculation of the difference between the average ΔCt values of the tumour samples for each target and the ΔCt value of the normal tissues for that target (ΔΔCt). The relative quantification value, fold difference (mean), is expressed as $2^{-\Delta\Delta Ct}$. *Normal tissue is standardised to baseline.*

2.3. Immunohistochemistry and immunofluorescence

For histomorphological analysis TRAIL-R1 antibody was provided by ProSci (CA, USA) and TRAIL was purchased from Abcam (Cambridge, UK). IFN-gamma and Fas-L were provided by BD Biosciences (Heidelberg, Germany), Fas, CD8 and CD56 antibodies were provided by Dako (Hamburg, Germany) and TNF-α was from R&D systems (Wiesbaden-Nordenstadt, Germany). Isotype control antibodies were purchased from eBioscience (San Diego, USA) and secondary antibodies used for immunofluorescence double staining and immunohistochemistry came from Jackson ImmunoResearch Laboratories Inc. (Suffolk, England). The secondary TRAIL-R1 antibody was a FITC-conjugated AffiniPure Donkey anti-rabbit IgG and the secondary CD8 antibody was a Cy3-conjugated AffiniPure Donkey anti-mouse IgG at a 1:200 dilution (Jackson ImmunoResearch).

The staining with TRAIL-R1, TRAIL, IFN-γ, Fas-L, Fas, CD8, CD56 and TNF-α was performed on serial cryostat sections of the 65 snap-frozen colorectal tumour specimens with neighbouring normal colon tissue and 12 normal colon specimens. In addition, we analysed TRAIL, Fas-L and TNF-α expression in the tumour cell line HT-29 (Promochem, Wesel, Germany) as controls for cytospins.

Serial cryostat sections (5 μm) were mounted on glass slides and fixed in acetone for 10 min and then dried for 5 min. Subsequently, the slides were incubated with the primary antibody or control antibody diluted in TBS plus 0.5% bovine serum albumine (BSA) overnight at 4 °C in a humidified chamber and with secondary FITC-conjugated (fluoreszeinisothiocyanat) antibody for 30 min at room temperature in a humidified chamber. The slides were subsequently incubated with the second primary antibody diluted in TBS plus 0.5% BSA overnight at 4 °C in a humidified chamber followed by incubation with secondary Cy3-conjugated antibody for 30 min at room temperature in a humidified chamber. Slides were counterstained with DAPI (4′,6-diamidino-2-phenylindoldihydrochlorid) (Sigma–Aldrich, Steinheim, Germany) and covered with polyvinyl-alcohol mounting medium (DABCO) (Sigma–Aldrich) and analysed using a Zeiss camera (Oberkochen, Germany). Photographed images using Metamorph software (Visitron Systems, Puchheim, Germany) package were imported into Microsoft Office Picture Manager.

For immunohistochemistry, the pre-treatment fixation of the slides was the same as described for immunofluorescence. After incubation with the primary antibody, we used a horseradish peroxidise (HRP)-conjugated AffiniPure Donkey anti-mouse or a Donkey anti-rabbit or a Donkey anti-goat IgG at a 1:200 dilution (Jackson ImmunoResearch). Slides were subsequently incubated for 5 min in DAB (3,3′-diaminobenzidine) (Biogenex, San Ramon, USA), counterstained with Haemalaun and mounted with Glycergel (Dako, Hamburg, Germany). The quantification of each immunoenzymatic staining of tumour cells as well as infiltrating immune cells in six individual magnified fields (400× magnification) for each staining sample was scored by cell counting. The magnified fields were representative for the whole tumour section. The result of the staining was expressed in percentages (%) positivity.

All values were expressed as mean ± SEM. The sections were evaluated by two independent blinded investigators separately and in case of discrepancies both evaluated the slide simultaneously and reached an agreement in their final assessment.

2.4. In situ detection of apoptosis

Apoptotic cells and bodies were detected by the terminaldeoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick-end labelling (TUNEL) method (ApopTag® Plus Peroxidase In Situ Apoptosis Kit, Chemicon, Planegg-Muenchen, Germany). In brief, the sections were post-fixed, and endogenous peroxidase was quenched with 0.3% hydrogen peroxide. The sections were equilibrated in terminal transferase buffer before the addition of the reaction buffer containing digoxigenin-dNTP oligonucleotide. The digoxigenin-dNTP-containing oligonucleotide extensions were detected by anti-digoxigenin-peroxidase conjugate diluted in a blocking agent, followed by staining with DAB. The sections were washed and counterstained with Haemalaun and mounted with Glycergel (Dako, Hamburg). For negative controls, slides were incubated with TUNEL reaction mixture without TdT (terminal deoxynucleotidyl transferase). For positive controls, sections of the female mammary gland were used. The apoptotic index (AI) was defined as the ratio of TUNEL-positive infiltrated cells to all counted infiltrated cells × 100. The number of stained cells was counted in at least ten 400 × high-power fields. Cells were defined as apoptotic if the whole nuclear area of the cell labelled was positive. The magnified fields were representative for the whole tumour section. The

result of the staining was expressed in percentages (%) positivity.

2.5. Determination of the apoptotic index

The apoptotic index (AI) was expressed as the ratio of the number of positively stained infiltrating immune cells to all immune cells in a specified area, and the result was expressed as percentage in each case. Positively stained tumour cells with morphological characteristics of apoptosis were identified using standard criteria, including chromatin condensation, nucleolar disintegration and formation of crescentic caps of condensed chromatin at the nuclear periphery.[14,15] To determine the AI *on infiltrating immune cells*, 10–25 representative areas without necrosis were counted for each sample with a light microscope (400× magnification). The magnified fields used were representative for the whole tumour section. The result of each staining was expressed in percentages (%) positivity. The sections were evaluated by two independent blinded investigators separately and, in case of discrepancies, both valuated the slide simultaneously and reached an agreement in their final assessment.

2.6. Statistical analysis

Statistical analysis was performed with MedCalc Software (Mariakerke, Belgium). All values were expressed as mean (SEM). The survival time was determined as the time from tumour resection to tumour conditional death and as the time from tumour resection to time of obvious recurrence. The overall survival time in association with AI and TRAIL-R1 expression *on infiltrating immune cells* was estimated by the Kaplan–Meier method. In all cases, the Kolmogorov–Smirnov test was applied to test for a normal distribution. To analyse differences in the overall/tumour-related survival among patients after successful (R0) curative surgical resection for CRC patients were divided into two subgroups (dichotomous variables). For univariate analysis the mean cut-off value for either high or low expressors was set at 8% for AI on infiltrating immune cells and CD8+ infiltrating immune cells, 15% for TRAIL-R1 on infiltrating immune cells and tumour cells, 25% for TRAIL on tumour cells and 13% for TRAIL on infiltrating immune cells.

Univariate analysis of significance for AI and TRAIL-R1 (on infiltrating immune cells) expression differences in survival curves was evaluated by a log-rank test. Multivariate analysis by using the Cox Proportional Hazards Model was performed on all the parameters that were found to be significant on univariate factors. Correlation between two variables was judged by the Pearson's correlation coefficient test.

Data were analysed using the Student's *t*-test when means of two groups were compared. A *p*-value of less than 0.05 was accepted as statistically significant.

3. Results

3.1. Identification and quantitative analysis of apoptotic tumour infiltrating immune cells

Tumour sections were first stained with H&E for localisation of immune cells in the tumour tissue as well as in the bound-

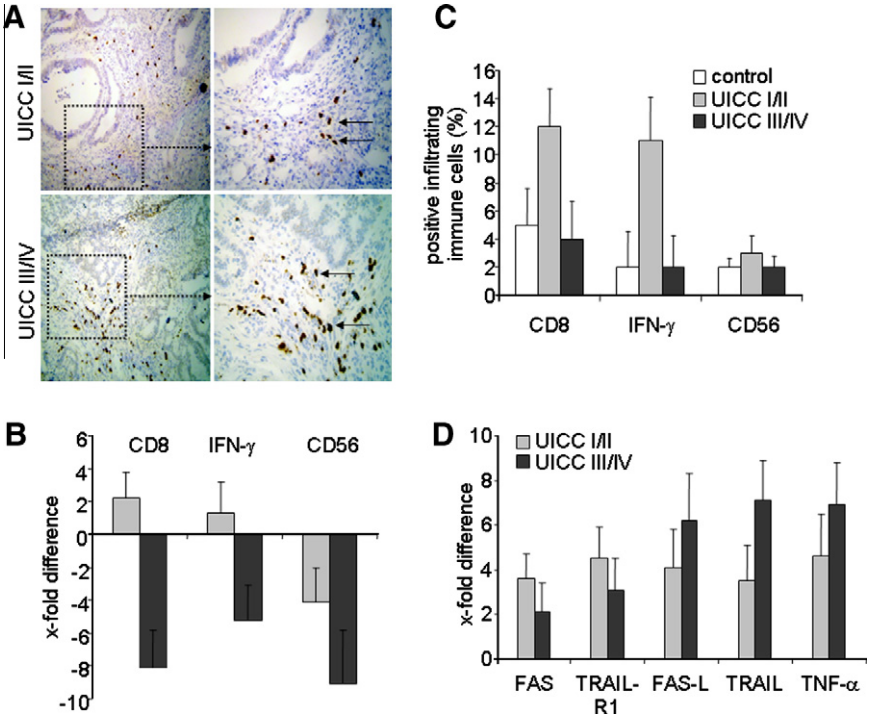

Fig. 1 – In situ detection of DNA fragmentation by TUNEL-staining in tumours (A), comparative RT-qPCR and immunohistochemical analysis of tumour infiltrating immune cells and IFN-γ (B and C), and (D) RT-qPCR analysis of apoptosis-related genes.

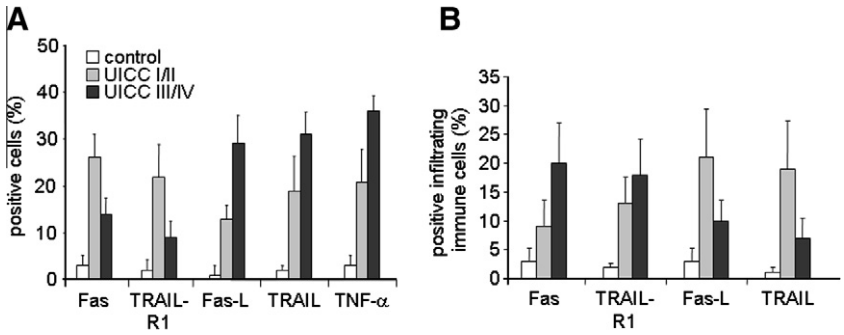

Fig. 2 – Immunohistochemical analysis of apoptosis-related proteins on tumour cells (A) and (B) tumour infiltrating immune cells.

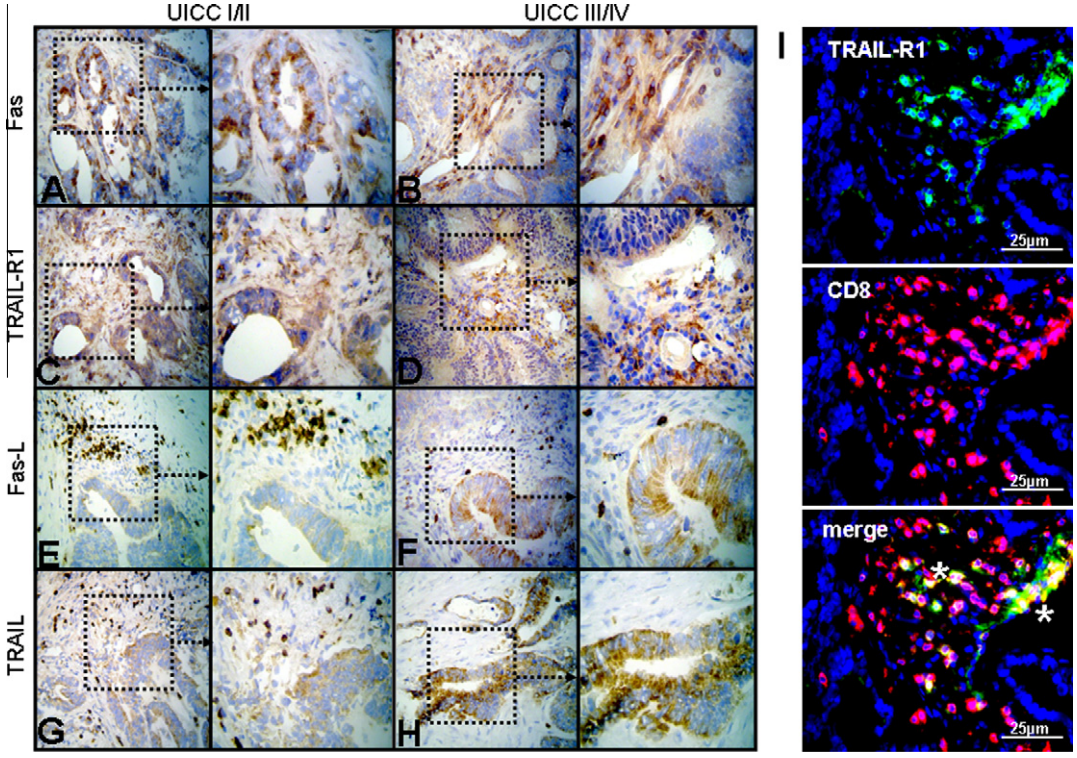

Fig. 3 – Representative images of immunohistochemical expression of apoptosis-related proteins in tumour infiltrating immune cells and tumour cells during tumour progression (A–H) and (I) a representative immunofluorescence double staining of TRAIL-R1 and CD8+ on tumour infiltrating immune cells.

ary areas. TUNEL-staining in the following sequential serial sections demonstrated TUNEL-positive cells in the identified areas of localised immune cells. This was shown in both areas, within the tumour and in the tumour border fields (Fig. 1A). The apoptotic index (AI) got by counting TUNEL-positive cells among all counted cells ranged from 1 to 16, with a mean of 4 ± 2.7 in early-stage tumours (UICC I/II) and 12 ± 2.6 in advanced-stage tumours (UICC III/IV) (normal tissue 0.3 ± 0.4; $p = 0.0001$ and $p < 0.0001$). These results indicate an increased rate of apoptosis in tumour infiltrating immune cells during tumour progression. Therefore, the AI *on infiltrating immune cells* was compared with the tumour-related survival of the analysed patients on an individualised basis (described below). Detailed analysis of AI *on infiltrating immune cells* is given in Table 1.

3.2. *RT-qPCR and immunohistochemical analysis for comparison of infiltrating immune cells and IFN- expression in the tumour*

RT-qPCR analysis showed an increased CD8 and IFN-γ gene expression in the early-stage tumours (UICC I/II) and the decreased expression in advanced-stage tumours (UICC III/IV) ($p < 0001$). In contrast, expression of CD56, indicative for NK cells, was significantly decreased at all stages ($p < 0.001$) (Fig. 1B).

The molecular findings in the tumour tissues were further analysed by immunohistochemical examination of tumour specimens from these patients. This demonstrated concordance with gene expression of CD8, IFN-γ and CD56 during tumour progression ($p < 0.001$) (Fig. 1C). Detailed analysis of CD8+ T cells is given in Table 1.

3.3. *Detection of apoptosis-related genes by RT-qPCR*

RT-qPCR analysis of the tumour tissues showed overall increased gene expression of Fas, Fas-L, TRAIL, TRAIL-R1 and TNF-α in all samples compared to normal controls. However, when compared between values at early and late stages of disease, gene expression of Fas and TRAIL-R1 decreased during tumour progression ($p < 0.0001$ and $p = 0.0002$, respectively) (Fig. 1D).

3.4. *Immunohistochemical and immunofluorescent analysis of apoptosis-related proteins on tumour infiltrating immune cells and tumour cells*

For further detailed analysis, we next examined expression of apoptosis-related proteins on both tumour infiltrating immune cells and tumour cells by immunohistochemistry with additional H&E staining.

First, we examined the expression of Fas and TRAIL-R1 on infiltrating immune cells and tumour cells during tumour progression. As shown in Figs. 2A and 3A & C, Fas and TRAIL-R1 expressions related to tumour cells were more pronounced in the early-stage tumours (UICC I/II) when compared to those in the late-stage tumours (UICC III/IV) (Figs. 2A and 3B & D) ($p < 0.0001$). Interestingly, Fas and TRAIL-R1 expressions related to infiltrating immune cells were in contrast increasingly observed in the late-stage tumours compared to those in the early-stage tumours and the normal tissue (Figs. 2B and 3B & D) ($p < 0.0001$).

We next examined the expression of Fas-L and TRAIL related to infiltrating immune cells and tumour cells during tumour progression. As shown in Figs. 2B and 3E & G, Fas-L and TRAIL expressions related to infiltrating immune cells were increasingly expressed in the early-stage tumours (UICC I/II) and less expressed in the late-stage tumours (UICC III/IV) ($p < 0.0001$) (Figs. 2B and 3F & H). Interestingly, both surface molecules, Fas-L and TRAIL expressions related to the tumour cells were increasingly expressed in the late-stage tumours compared to those in the early-stage tumours and the normal tissue (Figs. 2A and 3F versus E, and H versus G) ($p < 0.0001$).

TNF-α expression was significantly increased in all the tumour stages, indicating a potential role during tumour progression (Fig. 2A) ($p < 0.0001$). Cytospins and western blots of the colon tumour cell line HT-29 were assessed to control specificity of Fas-L, TRAIL and TNF-α expressions (data not shown).

Finally, examining tumour-infiltrating immune cells with serial immunofluorescence double staining plus additional H&E staining strongly indicated TRAIL-R1 expression in most of the patient tumours with demonstrated apoptotic events in the immune cells. TUNEL-positive immune cells in the tumours were detected in the next adjacent sections, with abundant TRAIL-R1-positive cells. These TRAIL-R1-positive immune cells were identified as preferentially CD8+ T cells (Fig. 3I).

A detailed analysis of TRAIL-R1 as well as TRAIL expression associated with infiltrating immune cells and tumour cells is given in Table 1.

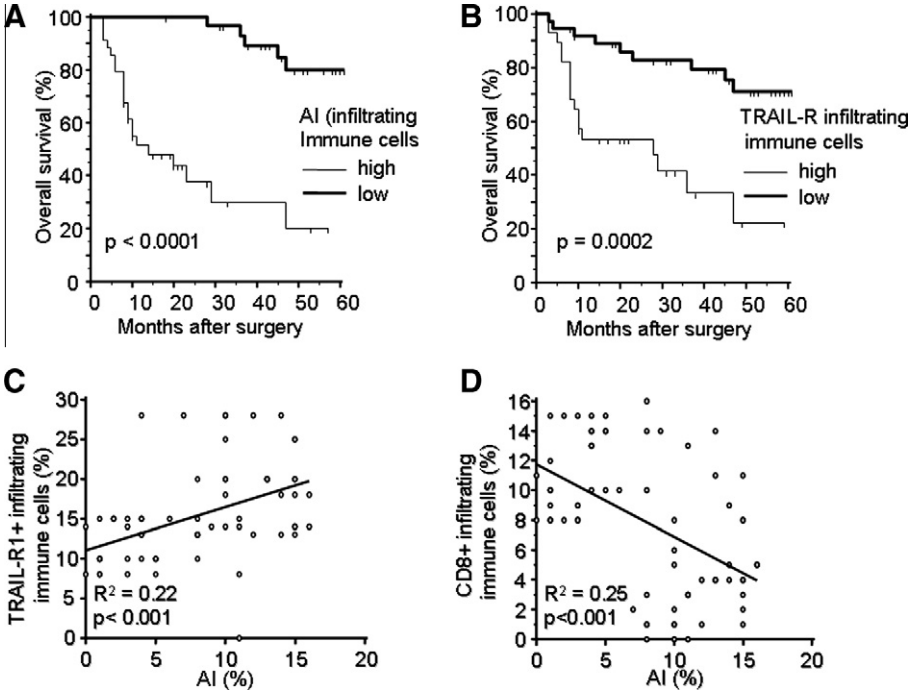

Fig. 4 – Tumour-related overall survival (Kaplan–Meier) of the analysed patients ($n = 65$) compared to differences in AI (A) and TRAIL-R1 (B) expression (infiltrating immune cells) and correlation between AI and TRAIL-R1 (C) and CD8+ T cells (D).

Table 2 – Multivariate analysis of prognostic factors of the patients (n = 65).

Variable	Unfavourable factor	Hazard ratio (HR)	95% Confidence interval (CI) of HR	p-Value
Depth of invasion	pT3/4	4.6613	1.6297–13.3319	0.004
LN	Positive	5.6286	1.6396–19.3223	0.006
AI (infiltrating immune cells)	High	7.2158	1.6906–30.7988	0.008
TRAIL-R1 (infiltrating immune cells)	High	2.1818	0.8705–5.4681	0.097
TRAIL (tumour)	High	4.0433	1.1178–14.6248	0.034

LN, lymph nodes metastasis and AI apoptotic index.

3.5. *Comparison of results from TUNEL-staining and TRAIL-R1 expression on infiltrating immune cells with patient survival*

For prognostic evaluation of apoptosis in infiltrating immune cells, tumour patients were divided into those with high and low apoptotic index (AI). Analysis of all patients showed that the mean overall survival at 5 years after curative resection of the primary tumour in the high AI and TRAIL-R1 immune cell subgroup was significantly lower than the survival of the low AI and TRAIL-R1 immune cell subgroup ($p < 0.0001$ and $p = 0.0002$, Kaplan–Meier graphs, Fig. 4A and B, and Table 1). Multivariate analysis was conducted among the significant variables of different clinicopathological variables in univariate analysis. Depth of invasion, N-status, AI *on infiltrating immune cells* and TRAIL-expression on tumour cells were found to be independent prognostic factors ($p < 0.05$, Cox–Mantel, Table 2) whereas TRAIL-R1 expression on infiltrating immune cells was found to be only a weak factor (Cox–Mantel, Table 2). Other variables including patient age, gender, grading, CD8+ T cells, TRAIL-R1 expression of tumour cells and TRAIL expression on infiltrating immune cells were not found to be independent predictors for survival. However, tumours with a high CD8+ T cell density, high TRAIL-R1 expression on tumour cells, and high TRAIL expression on the immune cells in the tumour were associated with significant prolonged survival (each $p < 0.0001$).

3.6. *Correlation of AI with TRAIL-R1 expression on tumour infiltrating immune cells*

To examine whether TRAIL-R1 expression corresponded with the AI *on infiltrating immune cells*, we stratified two different groups according to percentages of expression of immunohistochemical analysis. Considering the AI as a continuous variable, regression analysis showed that the AI directly correlated with TRAIL-R1 expression on tumour infiltrating immune cells ($R^2 = 0.22$, $p < 0.001$, $n = 65$; $r = 0.47$) (Fig. 4C). Similarly, an indirect correlation was found between the AI *on infiltrating immune cells* and the counted number of CD8+ T cells ($R^2 = 0.25$, $p < 0.001$, $n = 65$; $r = -0.50$) (Fig. 4D).

4. Discussion

Expression of apoptosis-related proteins on tumour cells may represent an efficient mechanism for a tumour to abrogate the host anti-tumour immune response. This hypothesis was recently proposed in several experimental studies, however the confirmatory clinical data is little-known yet. Our findings from the morphological and molecular analysis in combination with outcome data of a series of patients with CRC support this hypothesis.

It is known that upregulated TNF-α expression by macrophages or tumour cells may promote inflammatory conditions leading to tumour progression.[2] Our results show elevated TNF-α expression profiles in the tumour, which may promote further tumour growth in an autocrine and paracrine manner. Other TNF family members like Fas-L and TRAIL can trigger programmed cell death in susceptible cells via respective cell surface receptors as well. In the present study, we found upregulated gene and protein expression of Fas-L and TRAIL to be strongly associated to the tumour cells in a stage-dependent manner. Fas-L and TRAIL are effector molecules of cell-mediated cytotoxicity that participate in the immune response against neoplastic cells.[16] Therefore, resistance of tumour cells to the apoptosis-inducing activity of the ligands could contribute to their escape from immune surveillance. Fas-mediated apoptosis is one of these tumour-inhibiting pathways that must be overcome by the neoplastic cell during carcinogenesis.[17,18] Several potential mechanisms for resistance to Fas-mediated apoptosis have been described in tumour cells, including downregulation of Fas protein expression, intracytoplasmic sequestration and failure of the receptor to translocate to the cell surface, production and secretion of a soluble form of decoy receptor, or mutations of Fas, especially in the death domain.[19] Our histomorphological findings indicated decreased Fas expression on the tumour cells during tumour progression whereas infiltrating immune cells increasingly expressed the molecule at the same time. The induction of apoptosis in Fas-expressing immune cells infiltrating the tumour via Fas/Fas-L interaction by tumour cells has been described as Fas-mediated counterattack by the tumour. Thus Fas/Fas-L signalling would help the tumour to induce cell death for instance in activated FAS-expressing lymphocytes like CD8+ cytotoxic T cells that infiltrate the tumour.[20,21]

As demonstrated in this study, stage dependently upregulated TRAIL expression that was strongly related to tumour cells in CRC may help the tumour to defeat infiltrating TRAIL-R1-positive immune effector cells. Thus our results support the hypothesis of a TRAIL-R1-mediated apoptotic depletion of such tumour infiltrating cytotoxic T lymphocytes in response to TRAIL expression by the tumour itself. In fact, our results indicate a TRAIL-mediated counterattack as we

observed significantly more TUNEL- and TRAIL-R1 positive infiltrating immune cells in high TRAIL expressing subgroups than in low TRAIL expressing subgroups although the expression of TRAIL-R1 by infiltrating immune cells did not reach statistical significance. Therefore, induction of apoptosis may be induced by another Ref.mechanism of apoptosis signalling as indicated by Fas/Fas-L.[21] However, our findings give raise to be careful with TRAIL-based therapies.[22–24] Additionally, high TRAIL expression on tumour cells was shown to be an independent prognostic factor *whereas* high TRAIL-R1 positive tumour-infiltrating immune cells was demonstrated to be a weak prognostic factor not reaching statistical significance.

Our results of an increased TRAIL/TRAIL-R1 signalling during tumour progression are in accordance with the findings reported by others from patients with UICC stage III colon cancer as well as gastric cancer.[7,25] Moreover there is evidence from our differentiated analysis for an increased expression of the TRAIL receptor among CD8+ T cells during tumour progression. Indeed, comparably more infiltrating immune cells have been shown to undergo apoptosis at late stages of the disease compared to early-stage cancers. However, it remains speculative from this observation whether CD8+ T cells among these infiltrating cells are becoming apoptotic. Nevertheless this seems to be crucial for the hypothesis of a tumour progression by a TRAIL-mediated counterattack *through the tumour* against activated CD8+ T cells. There is evidence from several experimental clinical data for this concept of a TRAIL-mediated induction of apoptosis in activated CTLs infiltrating the tumour tissue.[5,6,26,27] Apoptosis in tumour infiltrating immune cells was suggestive to be relevant for the overall survival of our tumour patients. In addition, it has been identified as a risk factor for the development of multiple liver metastases and as an independent predictor of poor survival in patients with colorectal cancer.[28] Our data are in accordance with these findings and are indicative of an apoptotic depletion of tumour infiltrating immune cells by TRAIL/TRAIL-R1 signalling. Observations in the present study may eventually have implications for the treatment of CRC patients, particularly as a human recombinant TRAIL is already used in first clinical trial as a potential anticancer agent.[23] However, this strategy depends on the expression of TRAIL receptors on the tumour cells which then could render them susceptible to TRAIL-induced apoptosis. Interestingly, receptors other than TRAIL-R1 as investigated herein have been described to have no prognostic value (TRAIL-R2) or do not transfer the apoptotic signal to the tumour cell (TRAIL-R3 and -R4) as they lack an intracellular domain or have a truncated intracellular domain. In addition, our results indicate that only 20–30% of the tumour cells in the early stages of the disease express TRAIL-R1, which decreases to around 10% in the late-stage tumours. This indeed implies less susceptibility of the tumour cells for their induction of apoptosis during tumour progression. Nevertheless the ability to induce apoptosis in tumour cells makes TRAIL, an attractive anticancer agent. The decrease in the expression of TRAIL-R1 related to tumour cells as demonstrated in this study during tumour progression might be explained by the observation that TRAIL death receptors undergo constitutive endocytosis, as most recently shown in breast cancer cells.[29] To summarise, our findings indicate an increased expression of apoptosis-inducing molecules on tumour infiltrating immune cells during tumour progression. Therefore, analysis of these molecules before targeting tumours by TRAIL and its death receptor-based therapies might be a crucial step in cancer therapy. The prognostic value of TRAIL-R1 expressed by cancer cells remains controversial yet; however our findings could be helpful for guiding the development of TRAIL and its death receptor-based therapeutic strategies.

Conflict of interest statement

None declared.

Ethics

The present study has been approved by the local Ethic Committees.

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