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ABSTRACT

Drug resistance is believed to cause treatment failure in patients with metastatic colorectal carcinoma (CRC). Resistance to chemotherapy can involve different processes, including apoptosis, whose extrinsic pathway is regulated by expression of death-inducing TRAIL-R1 and -R2 and inhibitory TRAIL-R3 and -R4 cell surface receptors. Therefore, we investigated whether variations in their expression could influence the response to 5-Fluorouracile (5-FU) in metastatic CRC. We analysed TRAIL R-1, -2, -3 and -4 expression by immuno-histochemistry in CRC, using tissue micro arrays, and found that concomitant low/medium TRAIL-R1 and high TRAIL-R3 expression in primary CRC is significantly associated with a poor response to 5-FU based first line chemotherapy and with shorter progression-free survival. Specifically, the median progression-free survival was 3.1 months (poor prognostic group) vs 10.1 in the good prognostic group. Thus, the combination of TRAIL-R1 and TRAIL-R3 expression might represent a predictive and prognostic factor of the response to 5-FU-based first line chemotherapy in patients with metastatic CRC.

Key words: TRAIL receptors, apoptosis, TRAIL-R1, TRAIL-R3, immuno-histochemistry, colorectal cancer; 5-FU, chemo-resistance.

1. Introduction.

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane protein that belongs to the tumour necrosis factor (TNF) superfamily. TRAIL activates apoptosis through the death receptors TRAIL-R1 (DR4) or TRAIL-R2 (DR5/KILLER) which carry cytoplasmic death domains. TRAIL also binds to two other membrane-bound receptors, TRAIL-R3 (DcR1/TRID) and TRAIL-R4 (DcR2/TRUNDD), which can not transmit the apoptotic signal because they lack a functional intra-cytoplasmic domain. Consequently, TRAIL-R3 and TRAIL-R4 are thought to protect cells from TRAIL-induced apoptosis by acting as decoy receptors and thus interfering with the binding of the pro-apoptotic TRAIL-R1 and -R2.[1] Importantly, unlike other TNF family members, TRAIL kills a wide variety of tumour cells with limited effects on normal tissues *in vivo*. [2,3] This finding has aroused a great interest in the development of TRAIL targeted-therapies and currently several clinical trials are been carried out in cancer patients.[4,5]

Colorectal cancer (CRC) is the second most frequent cause of cancer death in Europe and United States. Chemotherapeutic agents can improve survival in patients with CRC, and 5-Fluorouracile (5-FU)-based chemotherapy, modulated with folinic acid, has long been a standard regimen. To enhance its efficiency in CRC patients, 5-FU is often administered in combination with other chemotherapeutic agents, such as oxaliplatin or irinotecan. Moreover, molecularly targeted therapies against epidermal growth factor receptor (such as cetuximab) and vascular endothelial growth factor (such as bevacizumab) have also shown promising effects in CRC. [6,7] However, despite the increasing therapeutic options, drug resistance remains a major problem for the treatment of this disease. As the ultimate goal of cytotoxic chemotherapies is to induce death of tumour cells, the presence of an imbalance between functional and decoy TRAIL receptors may be one of the mechanisms involved in the

development of drug resistance. Preclinical data have shown that the use of TRAIL in combination with chemotherapeutic agents, such as 5-FU, CPT-11 or doxorubicin, sensitises CRC cells to apoptosis [8,9], possibly through over-expression of TRAIL-R2. [10] Conversely, over-expression of TRAIL-R3 or -R4 has been correlated with resistance to apoptosis.[11,12] Therefore, *in vivo* analysis of TRAIL receptors' expression in CRC tumour samples might be of clinical relevance. To this aim we analysed, using tissues micro arrays, TRAIL-R1 to -R4 expression in metastatic CRC of patients treated with 5-FU based chemotherapy as first line treatment. We then correlated these findings with response to treatment, disease progression and survival.

2. Patients and methods

2.1. Patients and treatment

For this study, we selected 60 patients with metastatic CRC who were enrolled in controlled trials to evaluate the effect of first-line 5-FU-based chemotherapy at the CRLC Val d'Aurelle-Paul Lamarque Cancer Centre in Montpellier, France, between 1991 and 2002. All our patients had a histological diagnosis of advanced colorectal adenocarcinoma with hepatic metastases either at the time of the diagnosis (i.e., synchronous disease) or after resection of the primary tumour (i.e., metachronous disease), and underwent surgery for primary CRC and/or hepatic metastases. Patients' and tumours' characteristics are summarised in Table 1. The age range was between 35 and 80 years (median 63 years). Thirty-six patients were males (60%) and 24 females (40%). Median follow-up was 78 months (range 1.6 – 146 months). We excluded from this study any patient previously treated with radiotherapy.

All patients received a first-line 5-FU based regimen modulated by folinic acid. Specifically, 42 patients were treated with LV5FU2, 12 with FUFOL and 2 with capecitabine. The bimonthly LV5FU2 regimen was administered during 2 consecutive days as a 2-hour infusion of leucovorin (200 mg/m^2) followed by a 5-FU bolus (400 mg/m2) and a 22-hour continuous infusion of 5-FU (600 mg/m^2). In the monthly FUFOL regimen, leucovorin (200 mg/m^2) was administered as a 15-min infusion followed by a 5-FU bolus (400 mg/m^2) over15 min administered during 5 consecutive days every 28 days. The treatment schedule was repeated every 3 weeks until appearance of disease progression, severe toxicity or death. Treatment evaluation was performed every 3 months by computer tomography scanner for tumour response and every cycle for toxicity. Patients were considered evaluable for response if they had received at least 3 chemotherapy cycles. Following standard World Health Organization (WHO) criteria [13] we classified their clinical response into one of the following categories: complete response (CR), partial response (PR), stable disease (SD) and progressive disease

(PD). We observed an objective response (CR+ PR) in 19 (32%) patients, SD in 21 patients (35%) and PD in 20 (33%). We then subdivided our population in two groups, non-progressive (CR+ PR + SD) and progressive (PD), for further analyses.

2.2. Tissue Micro Array (TMA) construction

Initially we selected tissue blocks which appeared to have enough material upon macroscopic inspection and stained some sections with haematoxylin-eosin to check for the presence of cancer tissue. Thus, we could mark, on each donor block, areas with primitive and/or metastatic CRC. From these selected areas we sampled, using a manual arraying instrument (Beecher Instrument, MTA1), 0.6-mm tissue cores that were used to construct two tissue micro arrays. The sampling consisted of three malignant cores from different areas of a single primitive and/or metastatic CRC that were placed at specific coordinates. After the arraying was completed, we cut 4 µm sections from each TMA block, and stained with haematoxylin-eosin one section to confirm the presence of tumour tissue within each spot. The spots of 53 primary CRC and 33 hepatic metastases had enough material to assess the expression of the TRAIL receptors and only 5 patients out of 60 had insufficient material on both TMA blocks. In these cases we used classical sections for immuno-histochemistry.

2.3. Immuno-histochemistry

We deparaffinised and rehydrated through graded ethanol solutions adjacent 4 µm sections that were then immersed sodium citrate buffer (10mM, pH 6) at °C for 40 min for epitope retrieval. After neutralisation of the endogenous peroxidase activity with 3% hydrogen peroxidase in methanol for 15 min, we incubated the sections with primary antibodies against the four TRAIL receptors for 90 min and then visualised the binding with DAKO Liquid

LSAB+ following the manufacturer's recommendations (Dako, Glostrup, Denmark) using the Dako autostainer (Dako, Glostrup, Denmark). We purchased anti-human TRAIL-R1 (clone AF347, 1:10 dilution), anti-human TRAIL-R3, (clone AF630, 1:20 dilution) and anti-human TRAIL-R4 (clone AF633, 1:100 dilution) from RnD Systems (Minneapolis, USA), and anti-human TRAIL-R2 (clone ALX-210-743, 1:10 dilution) from Alexis (Switzerland). The specificity of these antibodies has been previously assessed by immunohistochemistry [14-16], by FACS [17] or by Western Blot.[18] We used diaminobenzidine (DAB, Dako) as chromogen and lightly counterstained sections with haematoxylin. Sections incubated only with the appropriate secondary antibody were used as negative controls.

2.4. Immuno-staining evaluation

We ranked each spot according to the percentage of positive cells from 10 to 100%. Continuous variables were subdivided into three equally-spaced quantiles (< 33% : low, 33-66% : medium, > 66%: high) that corresponded to the percentage of positive cells for each TRAIL receptor, whatever the intensity. Staining was separately evaluated for each spot. For triplicate cores, data were consolidated into a single score: the percentage value corresponded to the mean of the percentages of the three spots. The pathologist who scored the specimens was blinded to all clinical information.

2.5. Statistical considerations

All information relating to patient characteristics, treatment, clinical and pathological factors, response to therapy and outcome were collected. Categorical variables were reported by frequencies and percentages. Univariate statistical analyses were performed using Pearson's Chi-2 test or Fisher's exact test if applicable. Survival time was measured as the time from the

beginning of chemotherapy to death. Patients alive at the time of analysis were censored at their last follow-up examination. Progression-free survival (PFS) was evaluated as the time from the beginning of chemotherapy up to progression or recurrence of the disease or death due to any cause. Survival rates were estimated according to the Kaplan-Meier method and the log rank test was used to assess differences between groups. Multivariate analyses for response to chemotherapy were performed by logistic regression using a backward stepwise selection procedure in order to evaluate the adjusted effects of the different variables. Cox's proportional hazards regression model using a backward stepwise selection procedure was used to investigate the impact of prognostic factors on PFS. All p values reported were two-sided. For all statistical tests, differences were considered significant at the 5% level. Statistical analyses were performed using the STATA 9.0 software.

3. Results

3.1. TRAIL receptors' expression in primary CRC and hepatic metastases

In primary CRC and hepatic metastases, the four TRAIL receptors were localised mainly in the cytoplasm (Figure 1A) and their expression was quite variable among the different samples. Therefore, we decided to rank our samples in three different classes (low, medium and high) according to the number of positive cells for each TRAIL receptor (Figure 1B). We then looked at the distribution of the three classes (Figure 2) and observed that, in both primary CRC and hepatic metastases, the decoy receptor TRAIL-R4, was more often classified as highly expressed than the pro-apoptotic TRAIL-R1($p<0.0001$) and TRAIL-R2 ($p<0.002$ and $p<0.014$ for primary CRC and HM respectively).

3.2. TRAIL receptors' expression and clinico-pathological data

As the patient cohort was relatively small, we chose to consider only two groups (i.e., low and medium TRAIL receptors' expression versus high TRAIL receptors' expression) in order to provide a stronger statistical power.

We did not find any significant association between the level of expression of each TRAIL receptor and age, gender, tumour site, tumour size or number of involved lymph nodes by univariate analysis. However, patients with low/medium TRAIL-R1 expression showed progressive disease (PD) during 5-FU-based chemotherapy more frequently than those with high TRAIL-R1 expression (45% vs 19%, $p=0.052$). We did not identify any other univariate association between TRAIL receptors' expression and PD, progression-free survival (PFS) or overall survival.

We then carried out a multivariate analysis to evaluate the effect of TRAIL receptors' expression, age, gender, tumour site, tumour size and number of lymph nodes on the response to chemotherapy and PFS. We did not find any correlation between low/medium or high TRAIL receptors' expression and objective response (CR+PR) to chemotherapy. However, when we compared progressive (PD) and non-progressive status (CR+PR+SD), we found that there was a significant higher risk of progressive disease in patients with concomitant low/medium TRAIL-R1 (OR = 8.95; p = 0.01) and high TRAIL-R3 expression (OR = 5.38; p = 0.034). Indeed, 70% of patients with low or medium TRAIL-R1 and high TRAIL-R3 expression developed progressive disease. Therefore, concomitant low/medium TRAIL-R1 and high TRAIL-R3 expression in primary CRC might represent a good predictor of non response to chemotherapy.

We obtained similar results for PFS, with hazard ratios of 2.48 (p = 0.011) in patients with low/medium TRAIL-R1 and 2.33 (p = 0.015) in those with high TRAIL-R3, respectively (Table 2), but did not observe any association between TRAIL receptors' expression and overall survival.

These results allowed us to construct a prognostic index (PI) which categorised patients into three prognostic groups (good, intermediate and poor) by multiplying the hazard ratios of each category. Accordingly, concomitant low/medium TRAIL-R1 and high TRAIL-R3 expression resulted in a hazard ratio of 2.48*2.33=5.78 and was associated with poor prognosis. The association of high TRAIL-R1 and low/medium TRAIL-R3 expression (reference category with a hazard ratio equal to 1) was associated with good prognosis. The intermediate prognostic category had a hazard ratio of 2.33 for high TRAIL-1 and high TRAIL-3 expression, or hazard ratio of 2.48 for low/medium TRAIL-1 and low/medium TRAIL-3 expression.

We then applied this PI to our patient cohort, and classified 8% of them into the good, 73% into the intermediate and 19% into the poor prognostic group. Accordingly, 0% of the good, 29% of the intermediate and 70% of patients of the poor prognostic group had PD during the first-line 5-FU chemotherapy (Table 3). Moreover, the poor prognostic group had a shorter median PFS than the intermediate/good prognostic group (3.1 months in comparison to 10.1 months; $p = 0.0034$) (Figure 3).

4. Discussion

Drug resistance remains a major issue in cancer treatment. In this study we explored the potential role of the expression level of the four TRAIL receptors in the response to 5-FU based chemotherapy in patients with metastatic CRC. Our results indicate that concomitant low/medium TRAIL-R1 and high TRAIL-R3 expression in primary CRC is significantly associated with a poor response to 5-FU based first line chemotherapy and with shorter progression-free survival. Then, by combining the levels of expression of TRAIL-R1 and TRAIL-R3, we constructed a prognostic index that allowed us to evaluate the risk of disease progression in our patients. To the best of our knowledge this is the first study showing the clinical relevance of both TRAIL-R1 and –R3 expression in stage IV CRC.

Controversial findings have been reported on the prognostic significance of TRAIL-R1 in CRC. A recent prospective study has shown an association between high TRAIL-R1 expression and shorter disease-free as well as overall survival time in stage III adjuvant-treated CRC patients.[19] On the contrary, another study demonstrated that high TRAIL-R1 expression is associated with a longer disease-free survival after curative resection of stage II and III CRC[20]. Several factors may account for the discrepancies between these studies, including differences in patients' group studied, in antibody sensitivities and in the grading system used to assess immunoreactivity. Strater and colleagues, [20] suggested that TRAIL-R1 expression could be useful to predict the response to adjuvant chemotherapy. This finding is consistent with our results and together they suggest that high expression of pro-apoptotic TRAIL-R1 is linked to a greater apoptotic potential in a therapeutic setting.

TRAIL-R1 and TRAIL-R2 have been studied in many malignant tumours, notably cervical and oesophageal carcinomas, because they are functional receptors of the apoptotic pathway and, therefore, potential therapeutic targets.[21-23] However, the decoy receptors TRAIL-R3 and TRAIL-R4 might also be important to predict the response and to explain the possible

resistance to a certain treatment.[12,24] Preclinical data have shown that TRAIL-R3 has a role in the protection of either normal or tumour cells through the induction of survival-signalling pathways or of altered apoptotic signals.[25-27]

Resistance to apoptosis has been evoked as a possible mechanism to explain the therapeutic failure of fluoropyrimidines. Indeed, it has been shown that silencing of the death-inducing TRAIL-R2 in tumours confers resistance to 5-FU.[28] On the other hand, 5-FU resistance in CRC cell lines could be overcome by inhibiting expression of the decoy TRAIL-R3 (manuscript in preparation). Moreover, the extracellular domains of both decoy and death-inducing TRAIL receptors can interact in a ligand-independent manner suggesting that pre-ligand assembly may account for the inhibitory effects of TRAIL-R3 and TRAIL-R4 in certain cell types.[29] Similarly, Clancy and colleagues, showed that inhibition of apoptosis depends on the formation of ligand-independent complexes between TRAIL-R2 and TRAIL-R4.[30] Taken together these results emphasise the role and the interaction of death and decoy TRAIL-receptors and their possible implication in resistance to 5-FU chemotherapy.

New and reliable prognostic and predictive markers for CRC response to treatment are required. Many proteins involved in different aspects of tumour development and treatment response have been extensively studied, including p53, metabolic enzymes and MMR (mismatch repair) proteins that play a role in microsatellite instability.[31,32] However, none of them is currently used or validated for routine chemotherapeutic management of patients. Our results demonstrate the clinical relevance of TRAIL-R1 and -R3 expression as predictive and prognostic marker in patients with metastatic CRC treated with 5FU-based chemotherapy. However, before using TRAIL-R1 and -R3 expression as a predictor for individually targeted therapy, our results need to be validated in prospective studies that will include a larger number of patients. Indeed, a methodical analysis of the expression level of the pro-apoptotic TRAIL-R1 in a variety of tumours could be interesting, considering the promising results

obtained in preclinical studies and phase I/II clinical trials with antibodies targeting TRAIL-R1.[33]

Conflict of interest statement

None declared.

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Figure legends

Figure 1 A. Localisation of TRAIL receptors (original magnification x 400). B. Expression pattern of the four TRAIL receptors in tissue micro arrays of primary CRC. Immuno-histochemical staining classes were defined as following: low < 33%, medium 33%-66% and high > 66% cells expressing that receptor (original magnification x100). C. Negative control: section was incubated only with the appropriate secondary antibody.

Figure 2 Distribution of TRAIL receptors' immunostaining classes in primary CRC (pCRC) and hepatic metastases (HM). Expression of each TRAIL receptor was indicated as following: low (black), medium (grey) and high (white).

Figure 3 Kaplan-Meier curves of PFS in metastatic CRC patients according to the prognostic index : poor (----) versus intermediate/good (—) prognostic groups.

Table 1. Clinico-pathological characteristics

Characteristics	*n*	%
Age		
< 60	25	42
≥ 60	35	58
Gender		
Male	36	60
Female	24	40
pT stage		
pT2	4	7
pT3	39	65
pT4	17	28
pN stage		
0	14	23
1	22	37
2	21	35
missing	3	5
Synchronous metastases		
yes	42	70
no	18	30
Tumour Site		
colon	51	85
rectum	9	15
Chemotherapy regimen		
LV5FU2	46	77
FUFOL	12	20
Capecitabine	2	3
Response		
Complete Response	1	2
Partial Response	18	30
Stable Disease	21	35
Progressive Disease	20	33

Table 2. Progressive disease during chemotherapy and progression-free survival according to TRAIL-R1 and –R3 expression in patients treated with 5FU-based first line chemotherapy.

Progressive disease	**Odds ratio**	**95% CI**	***p***
Low/medium TRAIL-R1	8.95	1.69-47.43	0.010
High TRAIL-R3	5.38	1.13-25.57	0.034
Progression-free survival	**Hazard ratio**	**95% CI**	***p***
Low/medium TRAIL-R1	2.48	1.22 – 5.01	0.011
High TRAIL-R3	2.33	1.18 – 4.63	0.015

Table 3 Progressive disease during first-line 5-FU-based chemotherapy according to the prognostic index

Prognostic index*	Number of patients	Progressive patients
Good	4	0 (0%)
Intermediate	38	11 (29%)
Poor	10	7 (70%)
Total	52	18

* Good = high TRAIL-1 and low/medium TRAIL-3; Poor = low/medium TRAIL-1 and high TRAIL-3; Intermediate = concordant TRAIL-1 and -3 expression (both high or both low/medium)

Figure 1

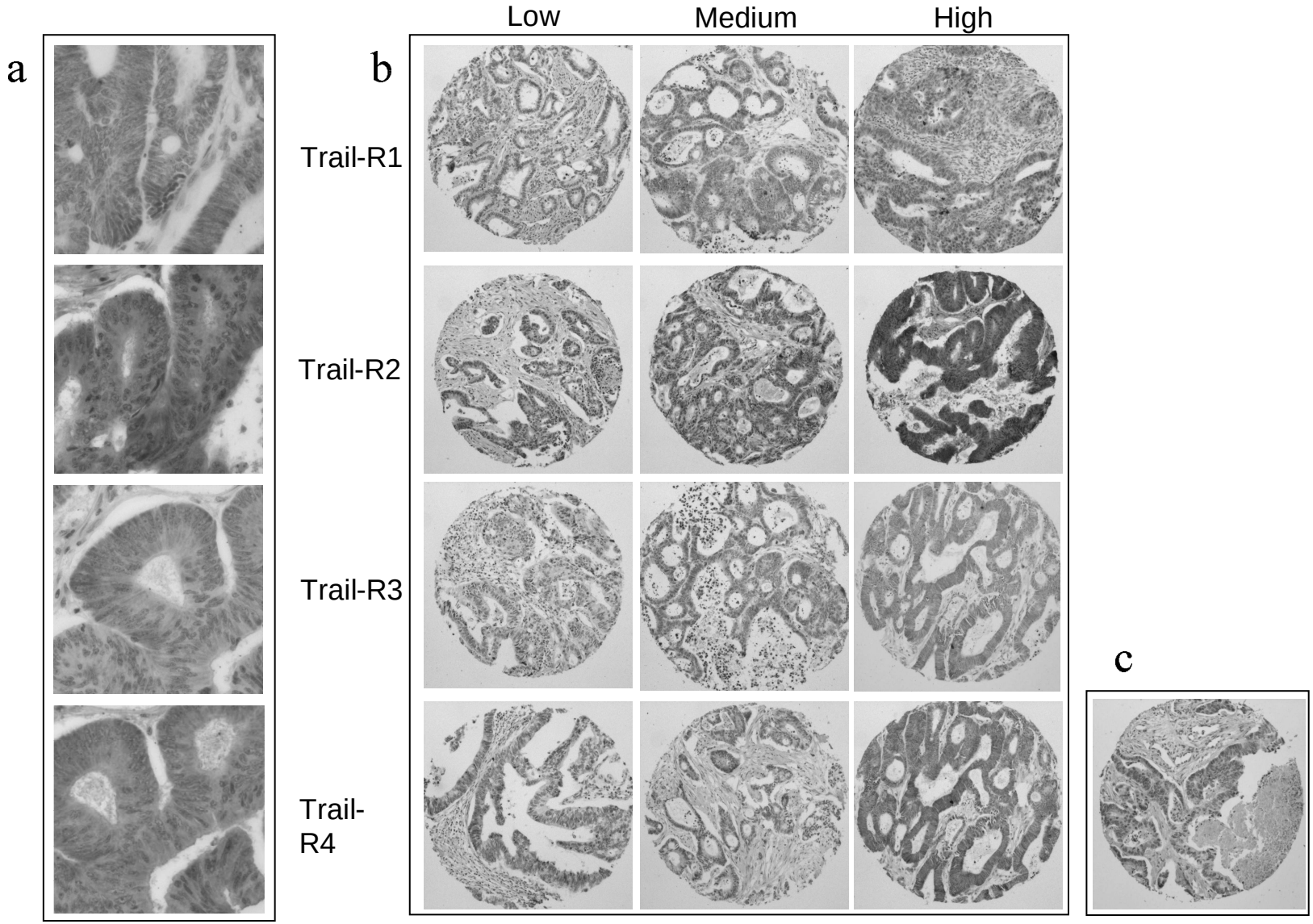
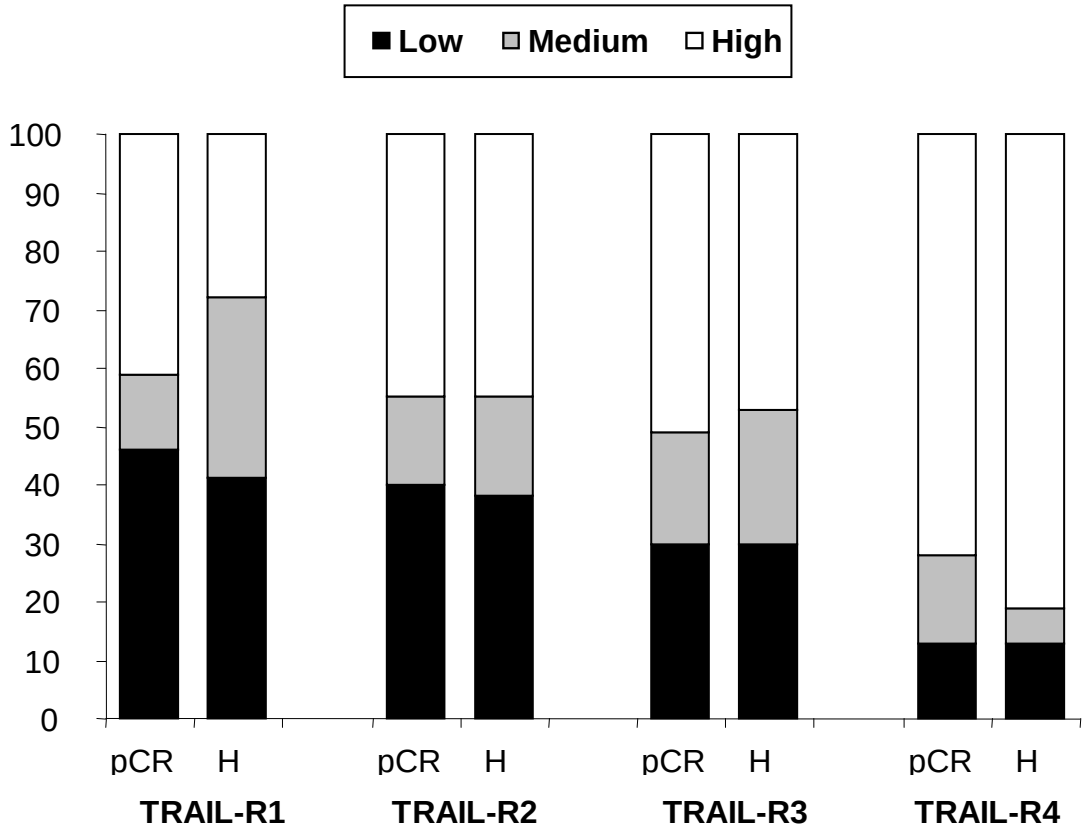


Figure 2


Low
Medium
High
100
90
80
70
60
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20
10
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pCR
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TRAIL-R1
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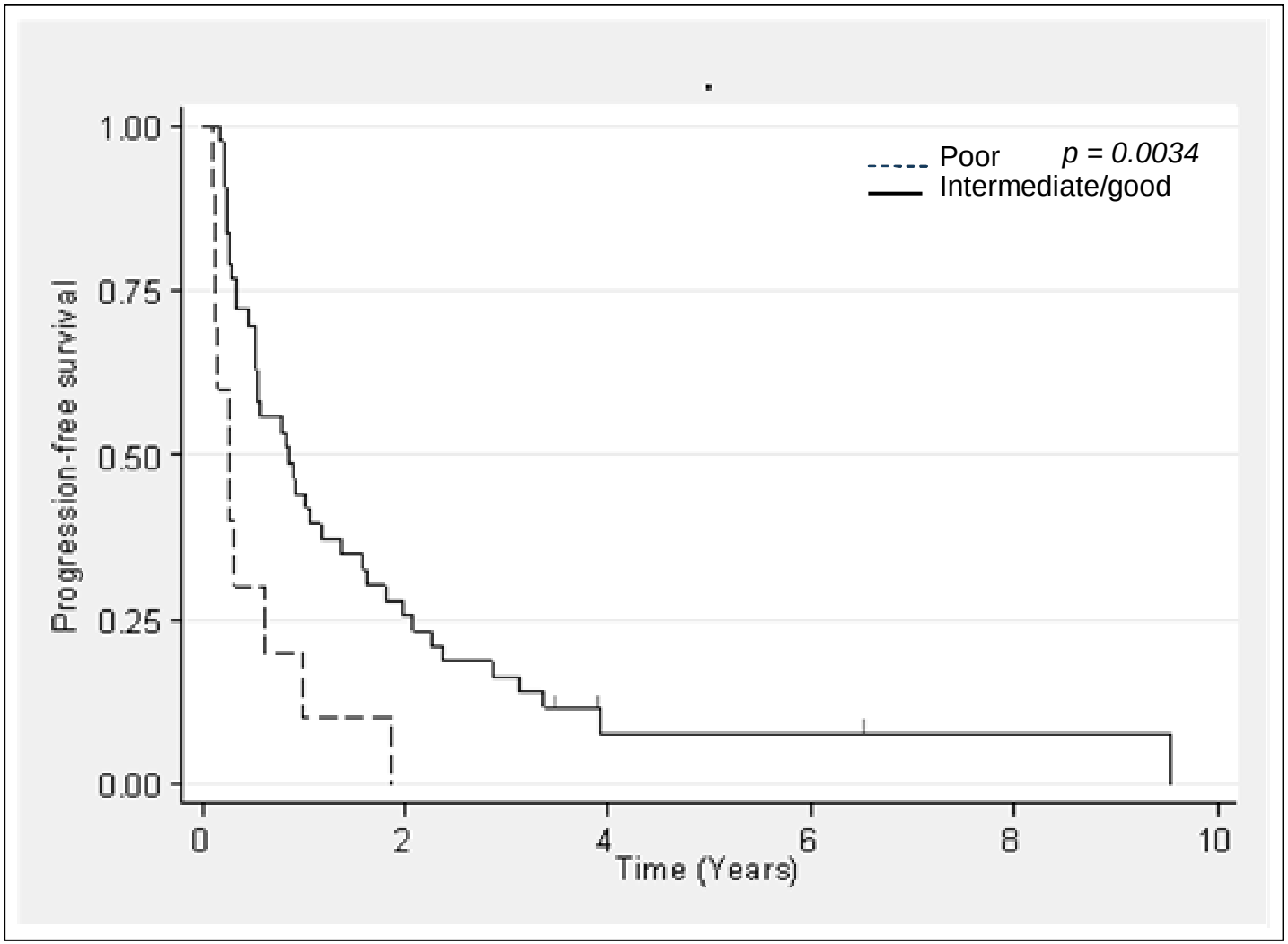
TRAIL-R3
TRAIL-R4

Figure 3


1.00
0.75
0.50
0.25
0.00
Progression-free survival
0
2
4
6
8
10
Time (Years)
Poor
Intermediate/good
p = 0.0034