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Association between preoperative plasma levels of tissue inhibitor of metalloproteinases 1 and rectal cancer patient survival: a validation study☆

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Abstract

The level of the tissue inhibitor of metalloproteinases 1 (TIMP-1) has previously been demonstrated to predict the survival of early stage colorectal cancer patients. The present study was undertaken to further validate plasma TIMP-1 as a prognostic marker in rectal cancer. Preoperative plasma from 352 rectal cancer patients were analysed using an immunoassay for TIMP-1. The TIMP-1 immunoassay demonstrated robustness and good reproducibility with low interassay coefficients of variation (CV). The rectal cancer patients had a mean plasma TIMP-1 level of 184 μg/l (standard deviation (SD): 70 μg/l). There were no significant differences in TIMP-1 levels between patients with Dukes' stage A, B or C disease, whereas Dukes' stage D patients had significantly increased TIMP-1 levels ($P < 0.0001$); however, high levels of TIMP-1 were not restricted to those with advanced disease. Univariate analysis demonstrated an increasing risk of mortality with increasing TIMP-1 levels Hazard Ratio (HR) = 2.9; 95% Confidence Interval (CI): 1.7–5.0; $P < 0.0001$). Including additional covariates, multivariate analysis identified plasma TIMP-1 as an independent prognostic marker (HR = 2.2; 95% CI: 1.2–4.1 ($P = 0.01$). This study showed a highly significant and independent association between preoperative plasma TIMP-1 levels and survival in rectal cancer patients, thus confirming our previous findings. Furthermore, the TIMP-1 immunoassay proved to be stable and reproducible in this confirmatory study.

Keywords: TIMP-1; Prognosis; Adenocarcinoma; Rectum; Plasma

1. Introduction

Current staging of patients with early colorectal cancer fails to adequately predict patient outcome, as 30% of patients with Dukes' stage B cancer will experience recurrence over a 5-year period whereupon the disease is fatal in most cases. Therefore, in early stage colorectal cancer (Dukes' stage A and B disease) there is a need to develop markers, which can be used to identify patients at high risk of disease recurrence, independent of the conventional stage classification. These patients might then be offered adjuvant therapy, while patients at low risk of disease recurrence could be spared such treatment and its adverse effects. Thus, with clinical guidance partly based on tumour marker testing, a tailored adjuvant therapy would be cost-effective and have the potential of reducing over- as well as under-treatment. The demand for the identification of new target molecules that potentially can be developed and tested as clinically applicable tumour markers is evident.

As described by Hayes and colleagues, acceptance of novel tumour markers in clinical settings requires thorough validation before being implemented into routine clinical use [1,2]. In order to propose guidelines on how promising markers progress from the laboratory into the clinic, Hayes and colleagues have introduced the tumour marker utility grading system: TMUGS. According to this system, a number of validation

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requirements are suggested which have to be fulfilled before the marker can be considered to have reached level of evidence I (LOE I), whereupon clinical implementation is feasible. Most tumour marker studies are LOE III, defined as retrospective studies where samples were not originally collected with the intent of testing the prognostic value of the marker of interest. The intermediate level—LOE II—is constituted by companion studies with prospectively collected specimens as part of a therapeutic trial with pre-established endpoints and evaluation of both the marker and the therapeutic intervention. Finally, LOE I studies are either (1) highly-powered prospective studies specifically addressing the issue of the utility of the marker or (2) an overview or meta-analysis of studies, each of which have a lower level of evidence.

We recently reported that total levels of tissue inhibitor of metalloproteinases 1 (TIMP-1) measured preoperatively in plasma are highly significantly associated with colorectal cancer survival: high plasma TIMP-1 levels predicting a shorter patient survival [3]. Of particular interest it was noted that preoperative plasma TIMP-1 was demonstrated to be a strong marker for patient outcome independent of clinicopathological parameters such as Dukes' stage and tumour location (colon vs. rectum) in a multivariate analysis, implying that plasma TIMP-1 measurements can be used to divide patients into distinct prognostic groups regardless of their stage of disease and tumour location.

In order to further validate the use of plasma TIMP-1 as an independent prognostic marker in patients with rectal cancer, we applied our TIMP-1 immunoassay to pre-therapeutically collected plasma samples from a cohort of 352 rectal cancer patients and correlated the plasma TIMP-1 values obtained with patient survival. Moreover, we further tested and validated our immunoassay for total TIMP-1 levels in this new set of plasma samples.

2. Patients and methods

2.1. Patients

In total, 352 consecutive patients with histopathologically-verified rectal cancer were included in the study. Blood samples (ethylene diamine tetra acetic acid (EDTA) vials) were prospectively collected between November 1994 and February 2001 at the first outpatient visit at the Department of Oncology, Lund University Hospital and before any treatment. The samples were centrifuged at 800 g for 10 min and the overlying plasma was transferred to a new tube and stored at −80 °C until use. The sampling procedure has previously been described in detail in Ref. [4]. 226 patients were males and 126 females and their median age was 68 years (range: 31–91 years). 79 patients were classified as Dukes' stage A, 128 as Dukes' B, 110 as Dukes' C and 35 as Dukes' D. For 346 patients, the grade of tumour differentiation was registered as either well (1), moderately (2) or poorly differentiated (3). The median time of observation was 43 months (range: 20–95 months; 1st–3rd quartile: 32–65 months) for the survivors and during this time 123 patients died (death from all causes). Of the 352 patients, total mesorectal excision (TME) of the primary tumour was performed for 286 (81%) as they were deemed to have resectable tumours. As part of the standard treatment regimen, these 286 patients also received short-course preoperative radiotherapy to a total dose of 25 Gy in 5 fractions. Of the remaining patients, 38 (11%) with primarily unresectable tumours were given prolonged radiotherapy (50 Gy in 25 fractions), while 28 (8%) patients having either small tumours or disseminated disease at the time of diagnosis were not given any radiotherapy. The Lund University Ethics Committee granted permission for the study. Patients' characteristics are given in Table 1. The data from this validation study including Swedish patients was compared with our initial Danish multicentre study where 255 patients with rectal cancer were included [3].

2.2. TIMP-1 assay

A sandwich immunoassay for the quantitation of total TIMP-1 levels (free and complexed forms) was applied to the preoperative plasma samples. In brief, the immunoassay consists of a polyclonal anti-TIMP-1 antiserum raised in sheep [5] for capture of antigen and a monoclonal anti-TIMP-1 IgG1 (MAC15) [6] for detection of the bound antigen. This antibody recognises both free and complexed TIMP-1 bound by the

Table 1
Patient characteristics ($n = 352$)

Dukes' stage	
A	79 (22%)
B	128 (36%)
C	110 (31%)
D	35 (10%)
Tumour differentiation grade[a]	
0	6 (2%)
1	5 (1%)
2	250 (71%)
3	91 (26%)
Gender	
Males	226 (64%)
Females	126 (36%)
Deaths	123 (35%)
Age (years)	68 (median) range 31–91

[a] Tumour differentiation grade: 0 = no data, 1 = well differentiated, 2 = moderately differentiated, 3 = poorly differentiated.

polyclonal antibody [7]. Finally, an alkaline phosphatase conjugated rabbit anti-mouse antiserum (Dako, Glostrup, Denmark) is used as the final layer enabling the kinetic rate assay. The TIMP-1 immunoassay has previously been rigorously validated and demonstrates low intra- and interassay coefficients of variation (CV) [8]. In the present study, two different plasma pools served as internal controls on each plate. The first plasma pool had an intermediate level of TIMP-1, the second a high content of TIMP-1. As part of the further validation of the immunoassay, interassay CV's for these two plasma pools were determined.

2.3. CEA and platelets

Preoperative serum samples were available for the carcinoembryonic antigen (CEA) analysis from 167 (47%) of the 352 rectal cancer patients. CEA was measured using an electrochemiluminiscent assay, CEA Elecsys (Roche Diagnostics). A cut-point of 5 μg/l for the distinction between high and low serum CEA was defined by the vendor. The platelet count was available for 197 patients.

2.4. Statistical analyses

The SAS® software package (version 8.2; SAS Institute, Cary, N.C., USA) was used to manage the patient data and for the statistical analysis. Preoperative plasma TIMP-1 values were considered continuous using $\log_e$ transformed values. The end-point for the survival analysis was death from any cause. The Kaplan–Meier method was used to estimate the survival probabilities, and the log rank test was used to test for equality of the strata. The Cox proportional hazard model was used for the multivariate analysis. Model validation was done graphically and using the methods described by Harrell and colleagues in Ref. [9]. Rank statistics were used to calculate correlation coefficients and to test the hypothesis on location. The level of significance was set at 5%. Significance testing was two-sided where appropriate.

3. Results

3.1. TIMP-1 assay validation

By the inclusion of two different plasma pools on each microtitre plate, the interassay variation of the enzyme-linked immunosorbent assay (ELISA) could be validated at different levels of TIMP-1. The mean (standard deviation (SD)) and median (range) concentrations for the two pools on 15 different plates were: 54 (5.8) μg/l, 55 (45–65) μg/l and 156 (13) μg/l, 155 (121–173) μg/l, respectively. The inter-assay coefficients of variation were 10.8% and 8.5% for the two different pools, respectively.

3.2. TIMP-1 in rectal cancer patients

All 352 patient plasma samples contained measurable TIMP-1 and the median (range) patient TIMP-1 level was 171 (80–618) μg/l; the mean (SD) level 184 (70) μg/l. When comparing plasma TIMP-1 levels in patients with different Dukes' stages, no significant differences in the TIMP-1 levels between Dukes' stage A, B or C rectal cancer patients were observed. However, patients with Dukes' D rectal cancer had significantly increased TIMP-1 blood levels ($P < 0.0001$). This is illustrated in Fig. 1, where plasma TIMP-1 values are depicted as a function of Dukes' stage. It can be seen that although Dukes' D patients on average had increased levels compared with patients in Dukes' stage A, B and C, high plasma levels of TIMP-1 were not restricted soley to those with advanced disease. In early stage rectal cancer patients (Dukes' A and B), high levels of TIMP-1 were also found. The median (range) and mean (SD) TIMP-1 levels in the different Dukes' stages were as follows: Dukes' A 167 (84–507) μg/l and 176 (71) μg/l, Dukes' B 172 (97–368) μg/l and 181 (54) μg/l, Dukes' C 167 (80–490) μg/l and 176 (62) μg/l and Dukes' D 205 (106–618) μg/l and 239 (112) μg/l.

Fig. 1 also depicts plasma TIMP-1 values as a function of Dukes' stage in the comparative Danish patient cohort ($n = 255$). Overall, plasma TIMP-1 levels were higher in the 352 Swedish rectal cancer patients. The median (range) TIMP-1 levels in the comparative Danish cohort was 126 (64–640); in the different Dukes' stages the median (range) TIMP-1 levels were: Dukes' A 118 (65–420) μg/l, Dukes' B 117 (75–510) μg/l, Dukes' C 124 (64–330) μg/l and Dukes' D 160 (81–640) μg/l. However, the distribution of plasma TIMP-1 between the different Dukes' stages were similar in the two cohorts of rectal cancer patients with increased levels in Dukes' stage D patients, but high levels also being found in the lower Dukes' stage patients.

When testing for an association between plasma TIMP-1 levels and patient age in the cohort of Swedish rectal cancer patients, a weak correlation between plasma TIMP-1 and age was found ($r = 0.12$; $P = 0.08$). This correlation was even lower than in our previous study, where a relatively weak association between plasma TIMP-1 and age of the rectal cancer patients was calculated ($r = 0.41$; $P < 0.0001$). No significant difference in plasma TIMP-1 levels was observed between males and females ($P = 0.36$) or when comparing different tumour differentiation grades ($P = 0.23$; grade 3 vs. 1 + 2) (Table 2).

3.3. CEA and platelets

The median (range; 1st–3rd quartile) CEA level in 167 of the 352 patients was 4.0 (0.5–8900; 2.0–11.0) μg/l. Using the predefined cut-off point of 5 μg/l, 103 patients

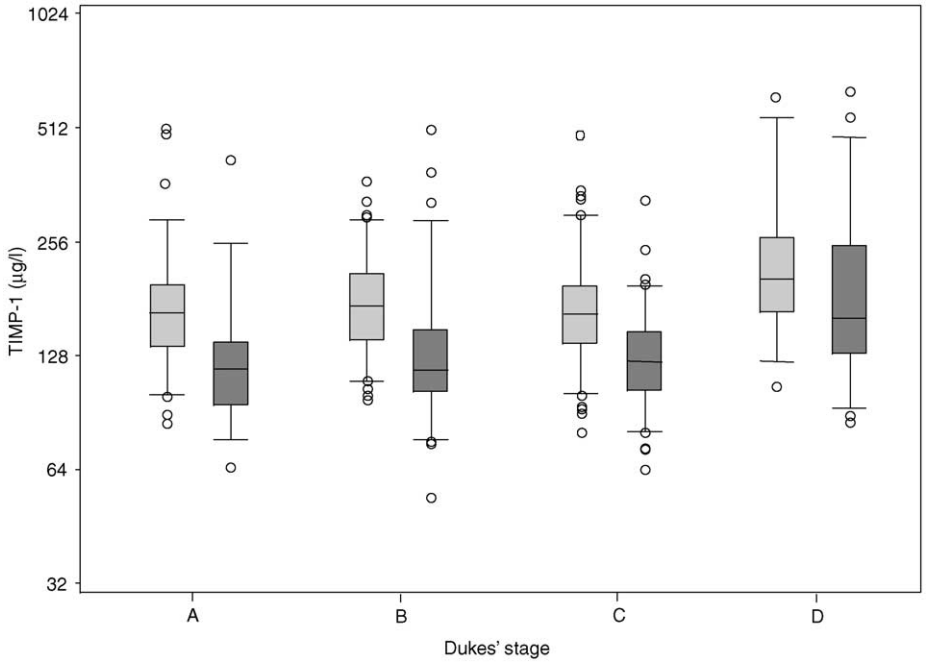

Fig. 1. Box plots demonstrating plasma tissue inhibitor of metalloproteinases 1 (TIMP-1) levels in relation to Dukes' stage in Swedish (dotted boxes) and Danish (dashed boxes) rectal cancer patient cohorts. Boxes indicate 25th, 50th and 75th centiles. Whiskers indicate 1.5× interquartile range (1st to 3rd quartile). Circles are outliers.

Table 2
Association of patients' characteristics with tissue inhibitor of metalloproteinases 1 (TIMP-1)

	P value	Spearman's correlation (*r*)
Dukes' stage[a]	<0.0001	
Tumour differentiation grade (n = 346)[b]	0.23	
Gender (male vs. female)	0.36	
Age (years)	0.08	0.12
Carcinoembryonic antigen (CEA) ($n = 167$)	0.86	−0.01
Platelet count ($n = 197$)	<0.0001	0.40

[a] Dukes' stage D significantly higher. Dukes' A, B and C are not significantly different.
[b] Tumour differentiation grade 3 vs. grades 1 + 2.

were defined as having a low CEA and accordingly 64 patients as having a high CEA. No association between plasma TIMP-1 and serum CEA was found ($r = -0.01$, $P = 0.86$) (Fig. 2a). In 197 patients where the platelet count was determined, the median (range) level was 282 (90–649) 10^9/l. However, a relatively weak significant association between plasma TIMP-1 levels and the platelet count was found ($r = 0.40$, $P < 0.0001$). This weak association is depicted in Fig. 2b, where the platelet count is plotted against plasma TIMP-1 levels. Table 2 summarises the associations between the measured parameters and the patient's characteristics.

3.4. Univariate survival analysis

When entering the TIMP-1 data in a univariate survival analysis, plasma TIMP-1 levels were significantly associated with survival ($P < 0.0001$). An increase in plasma TIMP-1 of 1 unit on the $\log_e$ scale corresponded to an estimated increase in the patient's Hazard Ratio (HR) of 2.9 (95% Confidence Interval (CI): 1.7–5.0). This association between plasma TIMP-1 levels and patient outcome can also be seen from Fig. 3, where the 352 rectal cancer patients were divided into three strata according to the level of TIMP-1 found in each individual plasma sample, such that each stratum yielded an equal number of patient deaths (death from all causes). The TIMP-1 levels in the three strata were: (I) TIMP-1 $\leqslant 164$, (II) $164 < \text{TIMP-1} \leqslant 213$, (III) TIMP-1 > 213 µg/l. From this Kaplan–Meier plot, it can be seen that plasma TIMP-1 was associated with a continuously increasing risk of mortality: patients with a higher TIMP-1 having a shorter survival.

In order to evaluate the prognostic value of plasma TIMP-1 in early stage rectal cancer, we stratified Dukes' stage A + B rectal cancer patients ($n = 207$) into two

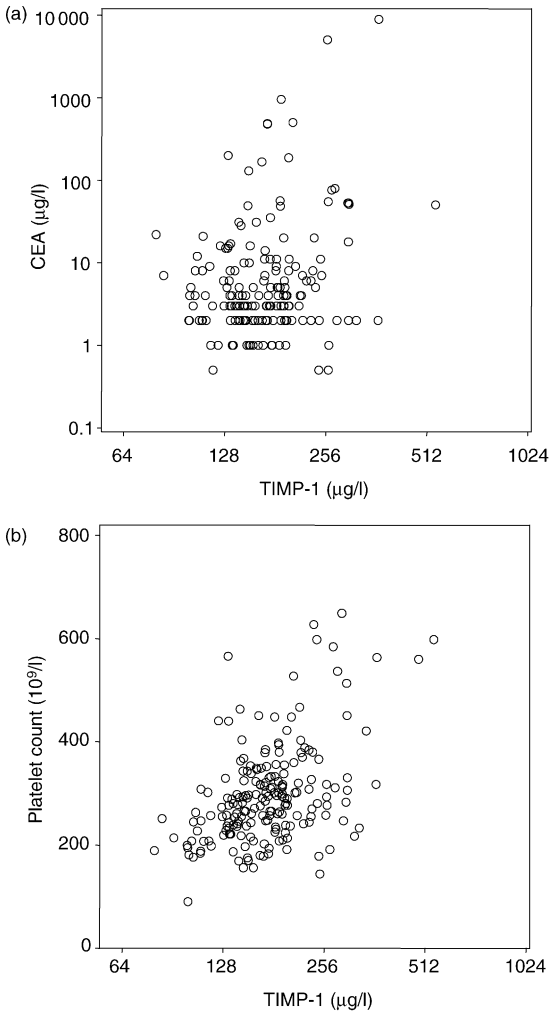

Fig. 2. Scatter plots of serum carcinoembryonic antigen (CEA) as a function of plasma TIMP-1 (Fig. 2a) and of platelet count as a function of plasma TIMP-1 (Fig. 2b).

groups according to their plasma TIMP-1 values being either above or below a chosen cut-off point of plasma TIMP-1, such that group I represents 70% of the early stage patients ($n = 145/207$) with the lowest plasma TIMP-1 levels and group II the remaining 30% ($n = 62/207$) with the highest TIMP-1 levels. This stratification was performed to reflect the average 5-year outcome of patients with early stage rectal cancer, where approximately 30% will relapse and subsequently die of their disease. It was found that TIMP-1 was significantly correlated with survival ($P = 0.01$) in these patients. The calculated plasma TIMP-1 cut-off point was 196 µg/l and a HR (95% CI) of 2.3 (1.2–4.3) was estimated reflecting the increase in hazard for a patient above the cut-off point compared with one in the group below.

3.5. Multivariate survival analysis

Including the variables Dukes' stage, tumour differentiation grade, age, gender and plasma TIMP-1 level, a Cox multivariate analysis of the survival data was performed with 346 eligible patients (for 6 patients data on tumour differentiation grade was lacking). Due to the low number of patients with CEA values and platelet counts, these two variables were not included in the multivariate analysis. Dukes' stage was found to be significantly associated with survival ($P < 0.0001$) with calculated HR's (95% CI) of 2.1 (0.9–4.7), 5.1 (2.4–11) and 21.9 (9.6–50) for Dukes' stages B, C and D with Dukes' A as the baseline group. Patient age was also found to be significantly associated with patient outcome ($P = 0.03$) with an estimated HR (95% CI) of 1.2 (1.0–1.4) per decade. Gender was not a significant parameter ($P = 0.67$): HR (95% CI): 1.1 (0.7–1.6). Tumour differentiation grade was demonstrated to be independently associated with patient survival ($P < 0.0001$) with a HR (95% CI) of 2.8 (1.9–4.1) when comparing a patient with a grade 3 tumour to a patient with a lower grade tumour (grade 3 vs. grade 1 + 2). Finally, plasma TIMP-1 was demonstrated to be independently associated with survival ($P = 0.01$) with an estimated HR (95% CI) of 2.2 (1.2–4.1), indicating the increase in hazard for patients differing by 1 unit on the $\log_e$ scale. The complete results from the multivariate analyses can be seen in Table 3.

4. Discussion

Several reports have demonstrated that TIMP-1—a naturally occurring inhibitor of the matrix metalloproteinases (MMP)—possesses alternative functions such as: stimulation of cell growth [10–12], inhibition of apoptosis [13–15] and involvement in malignant cellular transformation [16,17]. These activities have recently been demonstrated to be functionally distinct from the MMP inhibiting property of TIMP-1 [13,18]. Consequently, we speculated that plasma levels of TIMP-1 might serve as a marker of tumour growth and/or aggressiveness in colorectal cancer. This hypothesis was further supported by reports of an increasing number of intestinal tumours in Min mice overexpressing TIMP-1 [15]. We recently found that total levels of TIMP-1 in preoperative plasma from colorectal cancer patients were significantly associated with patient outcome and that high levels of plasma TIMP-1 predicted a short survival, independent of other clinicopathological parameters such as Dukes' stage, tumour location, age and gender [3]. In order to further validate the potential usefulness of plasma TIMP-1 as a tumour marker in accordance with the TMUGS-guidelines [2], we proceeded to measure total levels of TIMP-1 in plasma from a new and independent patient cohort. In addition, such a study enabled us to further evaluate the stability and reproducibility of our TIMP-1 immunoassay in a new set of plasma samples.

Table 3
Multivariate survival analysis

		P value	HR (95% CI)
Dukes' stage	B vs. A	0.07	2.1 (0.9–4.7)
	C vs. A	<0.0001	5.1 (2.4–11)
	D vs. A	<0.0001	22 (9.6–50)
Tumour differentiation grade	3 vs. 1 + 2	<0.0001	2.8 (1.9–4.1)
Gender	male vs. female	0.67	1.1 (0.7–1.6)
Age	decades	0.03	1.2 (1.0–1.4)
TIMP-1	1 $\log_e$ unit	0.01	2.2 (1.2–4.1)

HR, Hazard Ratio; 95% CI, 95% Confidence Interval.

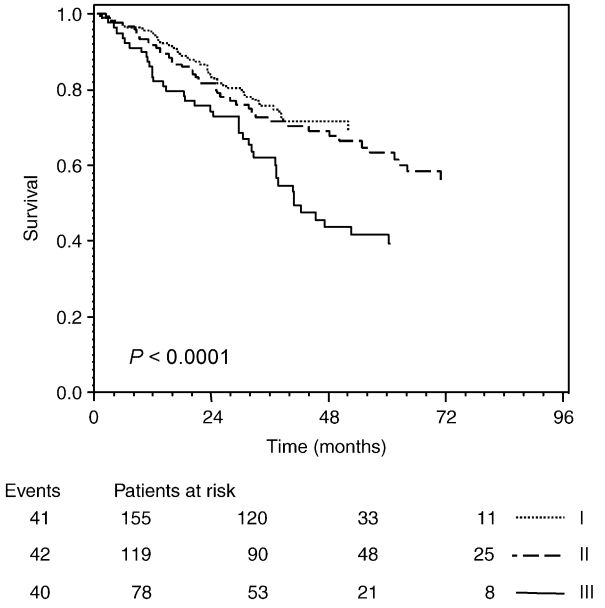

Fig. 3. Overall survival of 352 rectal cancer patients divided into three strata according to plasma TIMP-1 values. The number of patients at risk at 0, 24, 48 and 72 months is shown below the figure for each stratum aligned under the corresponding time point. The number of deaths (death from all causes) for each stratum is shown to the left. The plasma TIMP-1 (μg/l) values in the three strata were: (I) TIMP-1 $\leqslant$164, (II) 164 < TIMP-1 $\leqslant$ 213, (III) TIMP-1 > 213.

This confirmatory study including plasma obtained preoperatively from 352 patients with rectal cancer confirmed our previous findings: (1) Total levels of preoperative plasma TIMP-1 are not significantly different in rectal cancer patients with Dukes' A, B or C disease. However, Dukes' stage D patients have significantly increased plasma levels of TIMP-1, although high TIMP-1 levels are not restricted to those with advanced stage disease. (2) Total levels of preoperative plasma TIMP-1 are significantly associated with rectal cancer patient survival; high TIMP-1 levels predicting a short patient survival. (3) In multivariate analysis, a significant association between plasma TIMP-1 and patient survival exists; an association demonstrated to be independent of other clinicopathological parameters such as Dukes' stage, tumour differentiation grade, age and gender. Hence, these findings, in an independent set of patients from Sweden, suggest that preoperative measurement of plasma TIMP-1 in rectal cancer may have clinical value by identifying high and low risk patients. Thus, early stage patients (Dukes' stage A or B) with high preoperative plasma TIMP-1 and therefore at a high risk of disease recurrence would be potential candidates for adjuvant chemotherapy in order to improve prognosis. Several new chemotherapy agents have recently been introduced in colorectal cancer treatment. An optimised use of prognostic markers in combination with pre-operative radiotherapy and randomised chemotherapy trials offers great potential to improve survival for rectal cancer patients. However, as described above, it has been shown that TIMP-1 may protect cells against apoptosis and it therefore cannot be excluded that patients with high plasma TIMP-1 levels may also show an increased resistance to apoptosis-inducing anti-cancer therapeutics. A prospective randomised clinical study is needed in order to examine this further.

A secondary, but important, goal of our present study was to establish the reproducibility of the TIMP-1 immunoassay, which previously has been demonstrated to be highly specific and sensitive for total TIMP-1 with no cross-reactivity with TIMP-2, TIMP-3 or TIMP-4 [8,19]. By inclusion of two separate plasma pools as internal controls on each plate, it was possible to show good assay reproducibility by demonstration of a low interassay variability, both within the present study and in comparison with our previous studies where the TIMP-1 immunoassay and the same internal controls samples have been used. Thus, the immunoassay for measurement of total levels of TIMP-1 meets the demands for an assay used for quantitation of a plasma marker from different cohorts of patients and donors.

A significant difference between our previous study and this study was that the measured plasma TIMP-1 levels in the rectal cancer patients were (on average) 35% higher than those reported by us previously in the Danish rectal cancer patient cohort [3]. Possible explanations for such differences could be found in pre-analytical variables or in variables connected to the analytical phase itself. Obviously, the most crucial analytical variable is the immunoassay used for marker determination. If the assay is unstable or demonstrates a poor reproducibility, the results will be highly variable. However, as stated above, our TIMP-1 immunoassay has been thoroughly tested and validated and shows good stability. At the preanalytical stage, variables such as sample type (e.g. serum, EDTA plasma, citrate plasma), blood collection procedures, sample preparation procedures (e.g. centrifugation) and storage of samples can cause large differences in the levels of the molecule of interest. However, TIMP-1 levels are not affected by repeated freezing and thawing of samples for up to 6 times (data not shown). Furthermore, differences in centrifugation velocity and duration during plasma preparation have been tested and did not result in divergent TIMP-1 levels in our TIMP-1 immunoassay (Holten-Andersen and colleagues, data not shown). Choice of sample type (serum vs. plasma) is of particular importance for TIMP-1 determination, since this protein is stored in α-granules in platelets from where it can be released upon platelet activation [20]. The sample type analysed in the present study was EDTA plasma as in our original report. However, the collection and preparation of the samples were done according to a strict protocol in our previous report [3], whereas the same procedure was not applied in the present study, which was conducted between 1994 and 2001 in Lund, Sweden. These differences in the collection and preparation procedures of the EDTA plasma samples could possibly explain the differences in the TIMP-1 levels. Thus, the levels should not be directly compared between the two studies. Rather, focus should be directed to the associations between plasma TIMP-1 levels and patient outcome and the correlation to the other clinicopathological parameters.

Few other investigators have reported on the measurement of preoperative blood levels of TIMP-1 in colorectal cancer patients. Nevertheless, the existing reports have shown similar distributions of TIMP-1: i.e. Dukes' stage D patients having increased levels compared with Dukes' A, B and C patients but high levels also being found in some early stage disease patients [21,22]. Differences in immunoassays and various sample materials (serum vs. plasma) may well explain the differences in TIMP-1 levels in these studies compared with the values previously reported by our group.

A new and significant finding of the present study was the inclusion of CEA and platelet count and the association of these two parameters with plasma TIMP-1. CEA, which is the most thoroughly investigated marker in colorectal cancer, was not correlated to TIMP-1 levels, whereas platelet count was only weakly, but significantly, associated with TIMP-1. TIMP-1 has been demonstrated to be present in large amounts in platelets from where it can be released upon platelet activation [20], but the weak correlation between TIMP-1 and platelet count in the present study suggests that TIMP-1 is not merely a reflection of platelet count. We can speculate that the level of platelet activation rather than the actual platelet count might be associated with plasma TIMP-1. However, we have no data on the activation of the circulating platelets in this study.

In our first report, 49% (standard error of the mean (SEM): 3%) of the included rectal cancer patients were alive after 36 months, whereas 70% (SEM: 3%) of the patients were alive at the same time point in the our present confirmatory study. Even when looking only at early stage disease (Dukes' stage A + B)—there were fewer Dukes' stage D patients in this confirmatory study—the survival at 36 months was 73% (SEM: 4%) in the initial study compared with 83% (SE: 3%) in this study. From a treatment point of view, the patient population of this report is essentially different from that of our initial study. In this confirmatory study containing patients enrolled from 1994 to 2001, surgical technique (TME) and the use of pre-operative radiotherapy has been introduced which contrasts with treatments given to patients in our previous study. In the present sample, most patients (81%) received preoperative radiotherapy and TME surgery and only a few received either prolonged radiotherapy (11%) or no radiotherapy (8%). In our previous study, no radiotherapy was given prior to conventional (non-TME) resection of the primary tumour. This difference in treatment strategies is likely the reason for the longer overall survival of the rectal cancer patients in the present study. This may also explain why the separation of the three patient groups divided by increasing plasma TIMP-1 in the Kaplan–Meier plots were not as distinct as in our initial report. Nevertheless, it is noteworthy that despite the improved treatment of the rectal cancer patients—preoperative radiation therapy and TME surgery—preoperative plasma TIMP-1 was still a strong and independent marker for patient outcome.

According to the TMUGS guidelines, the next step would be to launch an appropriate prospective study where the benefit of using preoperative plasma TIMP-1 values in the clinical decision-making process (with regard to adjuvant therapy) is assessed. The study could be designed either as a single, high-powered, prospective, controlled study with the primary objective of testing the marker or a similar prospective study where

the primary goal could be the testing of a therapeutic hypothesis and secondly the marker (LOE I and II, respectively, in the TMUGS) [1]. Endpoints should include overall and disease-free survival, quality of life and cost-effectiveness.

An important remaining part of the final validation process of plasma TIMP-1 as a marker for rectal cancer patient survival is an external quality assurance of the immunoassay. Such a validation should establish low inter-laboratory variation and ensure reliable and comparable results across different borders. This would require the availability of large amounts of assay reagents produced under standardised conditions and used according to a strict protocol.

Another interesting future study would be the measurement of free (non-complexed) levels of TIMP-1 in preoperative plasma samples from colorectal cancer patients with the aim of relating these values to patient survival. As for another established tumour marker—prostate specific antigen (PSA)—measurement of specific sub-fractions of the molecule such as free or complexed forms, may reveal additional clinical information. As TIMP-1 can exist in either free unbound form or in complex with MMPs, we have recently reported on the development of an immunoassay for the quantitation of free TIMP-1 in human plasma and described normal reference ranges in a healthy donor population. Furthermore, we reported on the free and total TIMP-1 levels in a smaller cohort of colorectal cancer patients [19]. However, as those patients were newly diagnosed, no clinical follow-up data was available. Thus, we aim to extend our studies of the different forms of TIMP-1 to include measurements of free TIMP-1 in preoperative plasma samples from colorectal cancer patients with evaluation of the potential additional prognostic information.

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References

1. Hayes DF, Bast RC, Desch CE, *et al*. Tumor marker utility grading system: a framework to evaluate clinical utility of tumor markers. *J Natl Cancer Inst* 1996, **88**, 1456–1466.
2. Hayes DF. Determination of clinical utility of tumor markers: a tumor marker utility grading system. *Recent Results Cancer Res* 1998, **152**, 71–85.
3. Holten-Andersen MN, Stephens RW, Nielsen HJ, *et al*. High preoperative plasma tissue inhibitor of metalloproteinase-1 levels are associated with short survival of patients with colorectal cancer. *Clin Cancer Res* 2000, **6**, 4292–4299.
4. Fernebro E, Madsen RR, Ferno M, *et al*. Prognostic importance of the soluble plasminogen activator receptor, suPAR, in plasma from rectal cancer patients. *Eur J Cancer* 2001, **37**, 486–491.
5. Hembry RM, Murphy G, Reynolds JJ. Immunolocalization of tissue inhibitor of metalloproteinases (TIMP) in human cells. Characterization and use of a specific antiserum. *J Cell Sci* 1985, **73**, 105–119.
6. Cooksley S, Hipkiss JB, Tickle SP, *et al*. Immunoassays for the detection of human collagenase, stromelysin, tissue inhibitor of metalloproteinases (TIMP) and enzyme-inhibitor complexes. *Matrix* 1990, **10**, 285–291.
7. Holten-Andersen MN, Brunner N, Maimonis P, Jensen V, Murphy G, Piironen T. Characterization of monoclonal antibodies to tissue inhibitor of metalloproteinases-1. *Journal of Clinical Ligand Assay* 2002, **25**, 87–90.
8. Holten-Andersen MN, Murphy G, Nielsen HJ, *et al*. Quantitation of TIMP-1 in plasma of healthy blood donors and patients with advanced cancer. *Br J Cancer* 1999, **80**, 495–503.
9. Harrell Jr. FE, Lee KL, Mark DB. Multivariable prognostic models: issues in developing models, evaluating assumptions and adequacy, and measuring and reducing errors. *Stat Med* 1996, **15**, 361–387.
10. Docherty AJ, Lyons A, Smith BJ, *et al*. Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. *Nature* 1985, **318**, 66–69.
11. Hayakawa T, Yamashita K, Tanzawa K, Uchijima E, Iwata K. Growth-promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. A possible new growth factor in serum. *FEBS Lett* 1992, **298**, 29–32.
12. Luparello C, Avanzato G, Carella C, Pucci-Minafra I. Tissue inhibitor of metalloprotease (TIMP)-1 and proliferative behaviour of clonal breast cancer cells. *Breast Cancer Res Treat* 1999, **54**, 235–244.
13. Guedez L, Stetler-Stevenson WG, Wolff L, *et al*. In vitro suppression of programmed cell death of B cells by tissue inhibitor of metalloproteinases-1. *J Clin Invest* 1998, **102**, 2002–2010.
14. Li G, Fridman R, Kim HR. Tissue inhibitor of metalloproteinase-1 inhibits apoptosis of human breast epithelial cells. *Cancer Res* 1999, **59**, 6267–6275.
15. Goss KJ, Brown PD, Matrisian LM. Differing effects of endogenous and synthetic inhibitors of metalloproteinases on intestinal tumorigenesis. *Int J Cancer* 1998, **78**, 629–635.
16. Hewitt RE, Brown KE, Corcoran M, Stetler-Stevenson WG. Increased expression of tissue inhibitor of metalloproteinases type 1 (TIMP-1) in a more tumourigenic colon cancer cell line. *J Pathol* 2000, **192**, 455–459.
17. Guedez L, Courtemanch L, Stetler-Stevenson M. Tissue inhibitor

of metalloproteinase (TIMP)-1 induces differentiation and an antiapoptotic phenotype in germinal center B cells. *Blood* 1998, **92**, 1342–1349.

18. Chesler L, Golde DW, Bersch N, Johnson MD. Metalloproteinase inhibition and erythroid potentiation are independent activities of tissue inhibitor of metalloproteinases-1. *Blood* 1995, **86**, 4506–4515.
19. Holten-Andersen MN, Christensen IJ, Nielsen HJ, *et al.* Measurement of the noncomplexed free fraction of tissue inhibitor of metalloproteinases 1 in plasma by immunoassay. *Clin Chem* 2002, **48**, 1305–1313.
20. Cooper TW, Eisen AZ, Stricklin GP, Welgus HG. Platelet-derived collagenase inhibitor: characterization and subcellular localization. *Proc Natl Acad Sci USA* 1985, **82**, 2779–2783.
21. Oberg A, Hoyhtya M, Tavelin B, Stenling R, Lindmark G. Limited value of preoperative serum analyses of matrix metalloproteinases (MMP-2, MMP-9) and tissue inhibitors of matrix metalloproteinases (TIMP-1, TIMP-2) in colorectal cancer. *Anticancer Res* 2000, **20**, 1085–1091.
22. Simpson RA, Hemingway DM, Thompson MM. Plasma TIMP-1—a marker of metastasis in colorectal cancer. *Colorectal Dis* 2000, **2**, 100–105.