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Serum biomarkers of cell death for monitoring therapy response of gastrointestinal carcinomas

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ABSTRACT

Purpose: Antitumour treatments are thought to exert their therapeutic efficacy mainly by induction of apoptosis in tumour cells. In epithelial cells, caspases, the key enzymes of apoptosis, cleave the intermediate filament protein cytokeratin (CK)-18 into specific fragments that are released into circulating blood and can be detected by a specific ELISA.
Experimental design: To investigate the use of CK-18 fragments as a potential biomarker for the treatment response, we examined the association of serum CK-18 levels and clinical response in 35 patients with gastrointestinal cancers.
Results: While both cleaved and total CK-18 levels were intrinsically elevated in tumour patients, they were further increased during 5-fluorouracil (5-FU)-based therapy. Importantly, the increased levels of CK-18 could discriminate between patients with different clinical response. Cancer patients with a partial response or stable disease revealed a significantly higher increase of cleaved CK-18 during chemotherapy as compared to patients with progressive disease.
Conclusions: Our results suggest that detection of circulating caspase-cleaved CK-18 might be a useful serum biomarker for monitoring treatment response and should merit further evaluation in larger patient groups.

1. Introduction

Gastrointestinal cancer deriving from colon, stomach or oesophagus remains a major health problem worldwide. For instance, colorectal carcinoma represents the second cause of death from cancer in western countries. The overall prognosis of gastrointestinal cancer depends on the tumour stage and remains poor in advanced disease with 5-year survival rates of 5–8%.[1,2] Improvement of the overall survival rate therefore remains a major goal of anti-cancer treatment. Progress in the palliative treatment of gastrointestinal cancers has mainly been achieved by the development of empirically designed chemotherapy protocols applying 5-fluorouracil (5-FU)/leucovorin combined with different cytotoxic drugs in an optimised time course.[3,4] However, gastrointestinal cancers often show either primary unresponsiveness or secondary resistance towards initial palliative chemotherapy, which requires modifications of the treatment protocol.

The therapeutic response is routinely evaluated by imaging methods (ultrasound, computer tomography) that,

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however, are time- and cost-intensive. The carcinoembryonic antigen (CEA) is not necessarily elevated in patients with gastrointestinal carcinomas and does not allow for monitoring treatment response.[5] Furthermore, serum biomarkers that could predict the chemotherapeutic outcome at an early stage are far from being established.[6] The lack of appropriate methods for closely monitoring the chemotherapy response can result in prolonged ineffective treatment of patients. This can not only cause enormous costs, but also result in unnecessary side-effects of patients who might otherwise benefit from alternative treatment regimens. Thus, there is a strong need for new strategies that allow for early identification of patients non-responding to a particular tumour treatment modality.[7]

Over the past years, a variety of studies have demonstrated that the effect of anti-cancer drugs is mainly based on the induction of apoptosis in tumour cells.[8,9] Apoptosis induced by anti-cancer drugs is usually mediated by the so-called intrinsic mitochondrial pathway.[10] A key event of this pathway is the mitochondrial release of cytochrome *c*, which leads to the activation of caspase-9 and the effector caspase cascade. Effector caspases, such as caspase-3 and caspase-7, cleave a variety of cellular substrates and subsequently mediate the morphological alterations of apoptosis. Among the different substrates of caspases are proteins with diverse biological functions including scaffolding of the cytoplasm and nucleus, cell adhesion, cell-cycle control and signal transduction, as well as proteins involved in DNA replication and repair.[11] Important substrates also include intermediate filament proteins of the cytokeratin family, such as cytokeratin-18 (CK-18), the cleavage of which contributes to cellular collapse and apoptosis.

As chemotherapy-induced tumour cell death is thought to be mainly mediated by apoptosis, biomarkers of apoptosis could eventually enable monitoring of anti-tumour treatment efficacy. However, although a number of biomarkers have been proposed, validated biomarkers for tumour cell death are not yet established.[12] We and others have previously demonstrated that caspase-cleaved CK-18 is released from apoptotic epithelial cells and can be detected by an ELISA in patient sera employing an antibody (M30) specific for the cleaved CK-18 fragment.[13–16] Moreover, when this assay is combined with a pan-specific CK-18 ELISA that detects the total release of CK-18, even different forms of cell death, such as necrosis and apoptosis, can be distinguished.[17–19] An important advantage of these CK-18 biomarkers is that they are strictly selective for epithelial cell death but do not detect apoptosis in cells of non-epithelial origin. Thus, the unwanted toxicity of anti-cancer drugs towards other cells and tissues, e.g. bone marrow cells, is not measured. These biomarkers could therefore be promising non-invasive tools for the specific measurement of chemotherapy-induced cell death in tumours of epithelial origin, such as gastrointestinal cancers.

Although measurements of CK-18 and its caspase-generated fragments have been validated *in vitro*, circulating forms of CK-18 as biomarkers of prognosis or treatment response in tumour patients have not yet been established. In the present study, we therefore investigated the use of serum CK-18 measurement and its correlation to the clinical response in patients with gastrointestinal carcinomas. We found that both cleaved and full-length CK-18 levels were intrinsically elevated in tumour patients and further increased in response to 5-fluorouracil-based therapy. Importantly, the increased levels of CK-18 could discriminate between patients with different clinical outcome. Cancer patients with a partial response or stable disease revealed a significantly higher increase of cleaved CK-18 during the course of chemotherapy as compared to patients with progressive disease. Our results indicate that caspase-cleaved CK-18 might be a useful serum biomarker for monitoring treatment response, which should therefore warrant its evaluation in larger prospective clinical trials.

2. Materials and methods

2.1. Patients

We investigated sera from 35 patients with metastasised gastrointestinal cancer, stage UICC IV (colon $n = 25$, stomach $n = 7$, oesophagus $n = 3$) during the course of one cycle palliative standard chemotherapy (Table 1). All patients received 5-fluorouracil (5-FU)/leucovorin (LV)-based therapy combined with oxaliplatin ($n = 14$), cisplatin ($n = 3$), irinotecan ($n = 15$), bevacizumab ($n = 1$), cetuximab ($n = 1$) or mitomycin ($n = 1$). Nine patients of the 5-FU/LV/irinotecan group and 1 patient of the 5-FU/LV/oxaliplatin group additionally received cetuximab. Three patients treated with 5-FU/LV/irinotecan and 6 patients treated with 5-FU/LV/oxaliplatin additionally received bevacizumab. The patients were randomly selected in a prospective manner (23 men, 12 women, mean age 60 ± 1 years, range 29–87 years). Treatment response was investigated by comparing two computer tomography (CT) analyses performed usually within 3–4 months (one cycle chemotherapy). The patients were divided according to the international Response Evaluation Criteria in Solid Tumour (RECIST) criteria into responders, i.e. patients with partial response (PR, $n = 7$) or stable disease (SD, $n = 15$) and into non-responders, i.e. patients with progressive disease (PD, $n = 13$). If not otherwise indicated, sera were collected immediately before drug application at the beginning (baseline, day 1) and the end (e.g. week 12) within one cycle of chemotherapy and stored at –20 °C. The study was performed according to the guidelines of the Ethics Committee of the Hannover Medical School.

2.2. Immunohistochemistry

Paraffin-embedded colon sections from patients with colorectal carcinoma and healthy control colon tissues were obtained from the Department of Pathology at the Hannover Medical School. Five micrometre sections were deparaffinised in xylene and rehydrated through graded concentrations of ethanol. After blocking of endogenous peroxidase with 3% v/v hydrogen peroxide in methanol, the sections were microwave treated in 10 mmol/L citrate buffer, pH 6, for 5 min at 750 W followed by another 15 min at 100 W. Subsequently, the sections were cooled at room temperature for 15 min and balanced in PBS for 10 min. After blocking of non-specific binding with 1% w/v bovine serum albumin

Table 1 – Demographic and clinical features of patients.

	Total	Partial response	Stable disease	Progressive disease
No of patients	35	7	15	13
Mean age ± SEM	60 ± 1	57 ± 4	61 ± 2	63 ± 3
Sex (% male)	66	86	53	69
No of colon cancer	25	2	13	10
No of stomach cancer	7	4	2	1
No of oesophagus cancer	3	1	0	2

(BSA) for 1 h, the sections were incubated overnight at 4 °C with the M30 monoclonal antibody (0.5 μg/mL in 1% w/v BSA; Roche Molecular Biochemicals, Penzberg, Germany), recognising the neoepitope formed by caspase cleavage of cytokeratin-18 (CK-18) or, alternatively, with the anti-cleaved caspase-3 antibody (1:200; Cell Signalling, Beverly, MA). After repeated washings in PBS, the sections were incubated with biotinylated secondary antibody solution for 30 min (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). After washing again, bound antibody was detected with an avidin–biotin complex reagent containing horseradish peroxidase for 1 hour (Vectastain ABC kit). The sections were then washed in PBS and stained in freshly prepared substrate solution (4 mg aminoethylcarbazole in 10 mL sodium acetate buffer, pH 4.9, 500 μl dimethylformamide, 0.03% v/v hydrogen peroxide) for 10 min. The reaction was stopped by extensive rinsing in demineralised water. The sections were subsequently counterstained with haematoxylin and mounted in glycerol/gelatine.

2.3. Serological detection of caspase-cleaved and total cytokeratin-18

For the quantitative measurement of the caspase-generated CK-18 fragment and uncleaved CK-18, we used the M30-Apoptosense and M65 ELISA kits (Peviva, Bromma, Sweden) as described previously.[14,18,19] The M30 antibody detects a neoepitope specific to apoptosis caused by caspase cleavage of CK-18 at aspartate 396.[20] The M65 antibody detects a common epitope present in the full-length protein as well as in the caspase-cleaved CK-18 fragment, and thus measures, in addition to apoptosis, intact CK-18 that is released from cells undergoing necrosis.[20] The ratio was performed by dividing total CK-18 through caspase-cleaved CK-18.

2.4. Statistical analysis

Statistical analyses (SPSS 15 Software; SPSS Inc., Chicago, IL) comparing the concentration of the different variables in the serum of patients with gastrointestinal carcinoma showing response (partial response or stable disease) or no response (progressive disease) to chemotherapy were performed by using the Mann–Whitney test. The predictive value of the respective assays was determined by receiver-operating characteristic (ROC) analysis. The statistical analysis was confirmed by a professional statistician. A p-value of less than 0.05 was considered significant. All assays were performed in duplicate.

3. Results

3.1. In situ detection of caspase activation in colon tissues from patients with colon carcinoma

In first experiments, we investigated caspase activation in colon tissues of treatment-naive patients with metastasised colon carcinoma compared to healthy control colon tissue. Colon tissues were analysed for caspase activation using an activation-specific antibody for caspase-3 and the monoclonal M30 antibody that detects a specific caspase cleavage epitope of cytokeratin-18 (CK-18) which is expressed by epithelial cells. Representative immunohistochemical analyses are shown in Fig. 1. Whereas almost no caspase-3 activation and caspase-cleaved CK-18 were observed in healthy colon tissues (Fig. 1A and C), patients with colon carcinoma showed increased caspase-3 activation and CK-18 cleavage (Fig. 1B and D).

3.2. Detection of caspase-cleaved and total CK-18 in serum of patients with gastrointestinal carcinomas

We next investigated caspase activity in the serum of untreated patients with colon and other gastrointestinal carcinomas (colon $n = 25$, stomach $n = 7$, oesophagus $n = 3$) by using the M30 ELISA that detects caspase-generated CK-18 fragments (Fig. 2A). Compared to healthy control individuals ($n = 11$), patients with gastrointestinal carcinomas ($n = 35$) showed significantly ($p < 0.01$) increased caspase-generated CK-18 fragment baseline levels in the serum (87.0 ± 10.7 U/L; range 50–163 U/L versus 178.1 ± 24.6 U/L; range 62–869 U/L). In addition, we measured the serum levels of both uncleaved and cleaved CK-18 using the M65 ELISA as a marker of overall cell death (Fig. 2B). Patients with gastrointestinal carcinomas showed significantly ($p < 0.05$) higher total CK-18 levels (584.1 ± 66 U/L; range 69–1572 U/L) compared to healthy control individuals (310.9 ± 62.6 U/L; range 156–665 U/L). In line with the data from other epithelial tumours,[13,21,22] these results therefore suggest that the circulating levels of both total and caspase-cleaved CK-18 are already elevated in untreated cancer patients.

3.3. Serological monitoring of caspase activation in the course of chemotherapy of patients with gastrointestinal carcinomas

The anti-cancer effect of chemotherapeutics is mainly based on the induction of apoptosis in tumour cells. We therefore

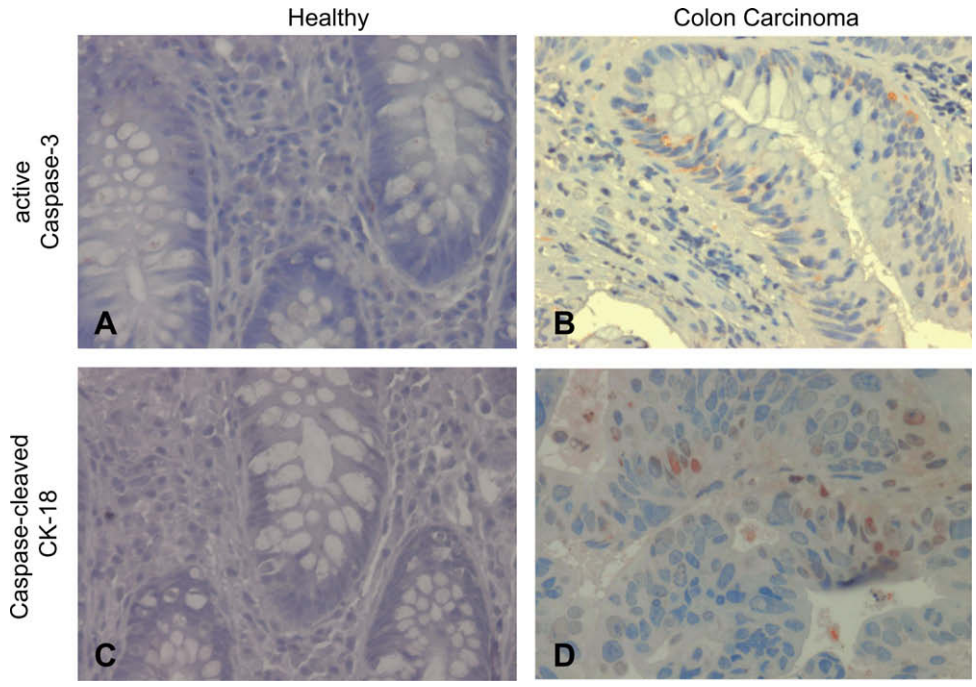

Fig. 1 – Immunohistochemical detection of caspase-3 activation (A, B) and caspase-cleaved CK-18 fragments (C, D) in a representative sample of healthy colon and colon carcinoma tissue. Healthy control tissues (A, C) showed no immunoreactivity, whereas cytoplasmic staining of both caspase-3 activation and CK-18 cleavage was detected in epithelial cells of colon carcinoma tissue (B, D).

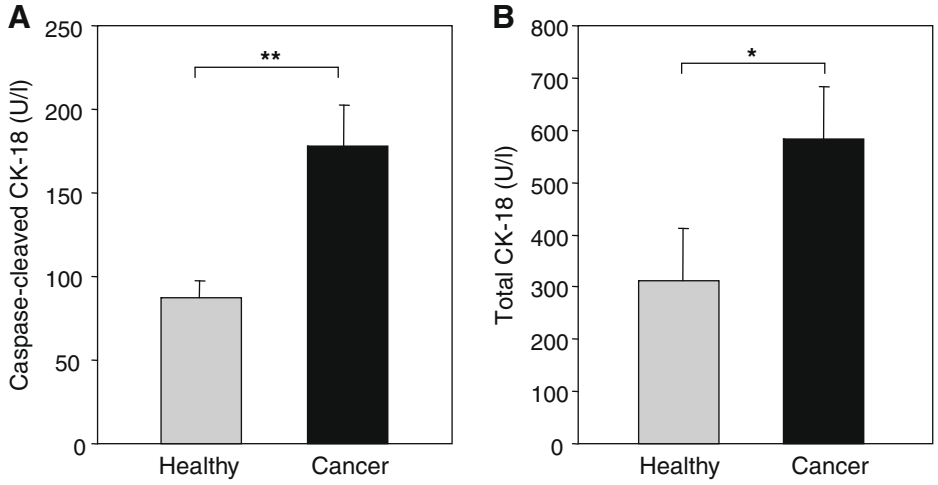

Fig. 2 – Serological detection of caspase-cleaved (A) and total CK-18 (B) baseline levels in patients with gastrointestinal carcinomas ($n = 35$) compared to healthy control individuals ($n = 11$). Caspase-cleaved and total CK-18 levels were significantly elevated in cancer patients compared to healthy control individuals ($^{*}p < 0.05$ and $^{**}p < 0.01$).

asked whether detection of caspase activation in sera of patients with gastrointestinal carcinomas might be a suitable biomarker for monitoring the treatment response. To this end, we analysed caspase-cleaved CK-18 in sera of patients with gastrointestinal carcinomas (colon $n = 25$, stomach $n = 7$, oesophagus $n = 3$) during palliative standard chemotherapy. Treatment response was assessed by comparing two computer tomography analyses. The patients were divided according to the international RECIST criteria into responders, i.e. patients with partial response (PR, $n = 7$) or stable disease (SD, $n = 15$) and into non-responders, i.e. patients with progressive disease (PD, $n = 13$). In initial experiments we measured M30 levels during one cycle before and during the first consecutive days of each drug application. However, no significant changes in caspase-cleaved CK-18 levels were found (data not shown). In further experiments we therefore measured M30 levels at the beginning (baseline) and end of drug application within one cycle, which revealed the most significant changes of cleaved CK-18 levels during chemotherapy (Fig. 3).

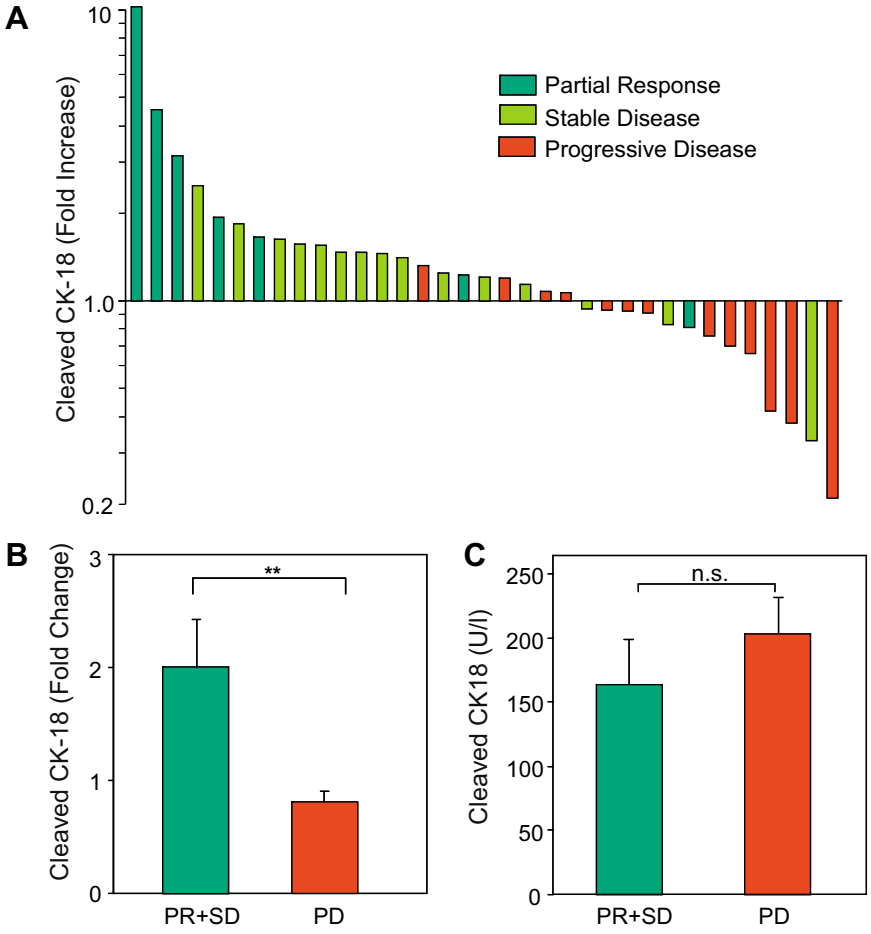

Fig. 3 – Serological detection of caspase-cleaved CK-18 during palliative chemotherapy of patients with gastrointestinal cancers. (A) Waterfall plot analysis of caspase-cleaved CK-18 serum levels in individual patients either responding (partial response and stable disease, *n* = 22) or non-responding (progressive disease, *n* = 13) to chemotherapy. Unchanged cleaved CK-18 levels in comparison to baseline levels were set as 1. The majority of patients with partial response or stable disease showed an increase of cleaved CK-18 levels, which was most strongly pronounced in patients with partial response. In contrast, most non-responding patients revealed no relevant changes of cleaved CK-18 levels upon drug treatment within one therapy cycle. (B) Statistical analysis of increased cleaved CK-18 levels during one cycle of chemotherapy. Responders showed a significantly higher increase in serum cleaved CK-18 levels compared to non-responders. (C) Baseline levels of cleaved CK-18 in patients responding (PR, SD, *n* = 22) or non-responding (PD, *n* = 13) to palliative chemotherapy. No significant differences in caspase-cleaved CK-18 baseline levels were found between responders and non-responders. PR = partial response, SD = stable disease, PD = progressive disease, n.s. = non-significant and $^{**}p < 0.01$.

In the waterfall analysis (Fig. 3A), treatment-induced changes in the individual patients are depicted as fold increase of caspase-cleaved CK-18. Interestingly, this analysis revealed that patients with partial response to chemotherapy (PR, *n* = 7) generally showed a stronger increase of CK-18 fragments than patients with stable disease (SD, *n* = 15). In contrast, the majority of patients with progressive disease (PD, *n* = 13) revealed a decrease of cleaved CK-18 levels in their serum at the end of the chemotherapy cycle. These findings were verified by statistical analysis (Fig. 3B) that demonstrated a significantly ($p < 0.01$) stronger increase in M30 levels (201 ± 43%) in patients responding to chemotherapy (PR, SD, *n* = 22) compared to patients with progressive disease who revealed a decline in cleaved CK-18 levels (81 ± 9%, *n* = 13) during chemotherapy. Moreover, our finding that responder and non-responder patients showed no significant difference in the baseline levels of cleaved CK-18 (Fig. 3C) indicated that serum changes of cleaved CK-18 were due to anti-cancer drug treatment.

3.4. *Comparison of cell death in patients with gastrointestinal carcinomas responding or non-responding to chemotherapy*

To analyse the mode of cell death-induced by chemotherapy, we conducted additional measurements of total soluble CK-18 in the sera. Similar as for caspase-cleaved CK-18, responder and non-responder patients revealed no significant differences in the baseline levels of total CK-18 (data not shown). We further calculated the ratio between total (caspase-cleaved and uncleaved CK-18) and caspase-cleaved CK-18, as detected by the M65 and the M30 ELISA, respectively. In line

with the increased M30 levels (Fig. 3A), during chemotherapy, most patients with partial response or stable disease revealed a decline of M65/M30 ratio during chemotherapy, whereas the opposite effects were detected in cancer with progressive disease (Fig. 4A). Accordingly, patients responding to chemotherapy showed a significantly ($p < 0.01$) lower ratio of M65/M30 (2.5 ± 0.2) at the end of the chemotherapy cycle compared to non-responding patients (M65/M30 ratio 3.9 ± 0.5; Fig. 4B). Similarly, responding patients showed a significant ($p < 0.01$) decline in the M65/M30 ratio (0.71 ± 0.09) upon anti-cancer treatment compared to patients with progressive disease who demonstrated an almost twofold increase (1.78 ± 0.32; Fig. 4C). Moreover, most patients (82.7%) revealed virtually no changes of alanine aminotransferase (ALT) levels during the course of chemotherapy (Fig. 4D). Only 10.3% of the patients showed an increase and 6.9% of the patients a decrease of ALT levels in the course of chemotherapy. These results indicate that caspase-cleaved and total CK-18 levels were presumably tumour-derived and not due to increased liver damage.

3.5. *Cut-off value of serological caspase activity to predict treatment outcome*

We then calculated the cut-off value of the relative change in serological caspase activation (M30 levels) that correctly predicts the treatment outcome with best-compromise sensitivity/specificity (predictive discriminating value). To this end, we performed a receiver-operating characteristic (ROC) plot analysis comparing patients responding (PR, SD) or non-responding (PD) to chemotherapy. Changes of caspase-cleaved CK-18 fragments above or below 111% correctly predicted the treatment outcome with a sensitivity of 82% and a specificity of 85% (area under the curve 0.878; confidence

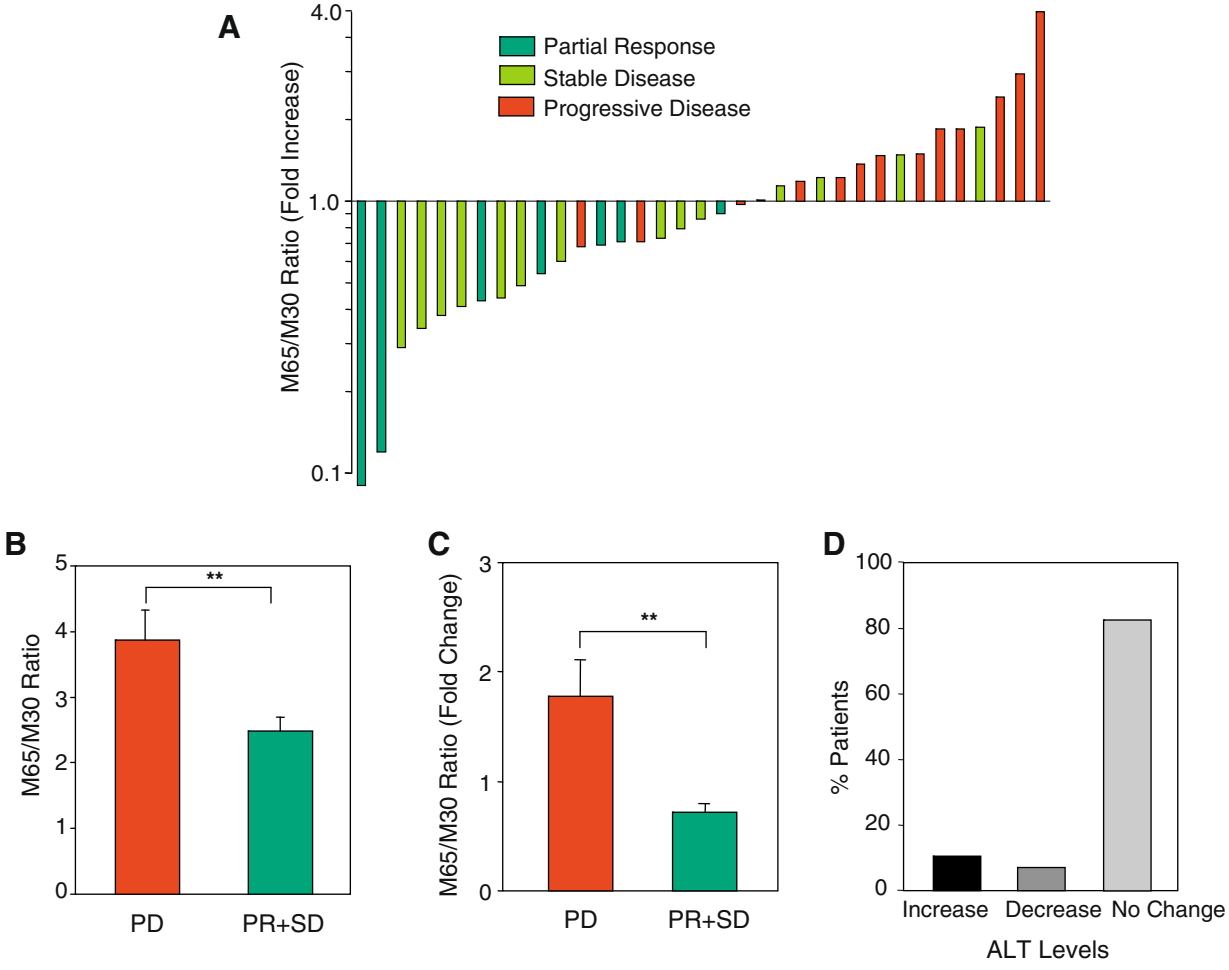

Fig. 4 – Serological detection of caspase-cleaved CK-18 (M30 ELISA) and of total (caspase-cleaved and uncleaved) CK-18 (M65 ELISA) in patients with gastrointestinal carcinomas either responding ($n = 22$) or non-responding ($n = 13$) to chemotherapy. (A) Changes of the M65/M30 ratio during chemotherapy in the individual patients are depicted by waterfall analysis. (B, C) Patients responding to chemotherapy showed a significantly ($p < 0.01$) lower M65/M30 ratio at the end of one cycle chemotherapy (B) and a significant ($p < 0.01$) decline of the M65/M30 ratio (C) induced by chemotherapy compared to patients with progressive disease. (D) Measurement of relative alanine aminotransferase (ALT) levels during chemotherapy in patients with gastrointestinal cancer ($n = 29$). 82.7% of the patients showed no changes of ALT levels, whereas 10.3% of the patients had increased and 6.9% of patients had decreased ALT levels. PR = partial response, SD = stable disease, PD = progressive disease and $^{**}p < 0.01$.

interval (CI) 95%, Fig. 5A). The therapy-induced changes of the M30 levels above or below the cut-off value of 111% for the individual patients are shown in Fig. 5B and C. In line with the previous data, the results demonstrate that patients with a partial response or stable disease mainly show an increase of the M30 levels, reflecting increased apoptosis. In contrast, patients with progressive disease predominantly show a decrease of the M30 levels during chemotherapy. By using this cut-off value for the prediction of the treatment response, only one false positive (Fig. 5B) and five false negative patients (Fig. 5C) were detected.

Compared to the serological detection of caspase-cleaved CK-18 fragments, most patients of our cohort showed no relevant elevation (⩽10 μg/ml) of carcinoembryonic antigen (CEA) at baseline (68% of responders and 72% of non-responders; Fig. 5D) and maintained normal lactate dehydrogenase (LDH) levels during chemotherapy (76% of responders and 75% of non-responders; Fig. 5E). These results demonstrate that monitoring of CK-18 serum levels during chemotherapy might reflect the clinical outcome. Prospective studies with larger patient cohorts are necessary to evaluate whether this biomarker might be useful for the prediction of treatment response in patients with gastrointestinal cancers.

4. Discussion

The overall response rates to palliative chemotherapy in patients with metastatic gastrointestinal cancers remain low. Although several second- and third-line treatment strategies have been established to improve treatment response, so far no serum biomarker for monitoring anti-cancer treatment responses exists. Thus, in advanced cancers the search for indicators of response to chemotherapy still represents a valuable effort to improve the therapeutic decision-making following failure to first-line therapies. The effect of chemotherapeutic drugs is mainly based on the induction of apoptosis in tumour cells. During apoptosis of epithelial tumour cells, CK-18 is cleaved by caspases and released by yet unknown mechanisms. Furthermore, overexpression of CK-18 in colon carcinoma and its association with tumour metastasis have been described,[23–25] suggesting that CK-18 could be a useful marker for investigating apoptosis in gastrointestinal cancers.

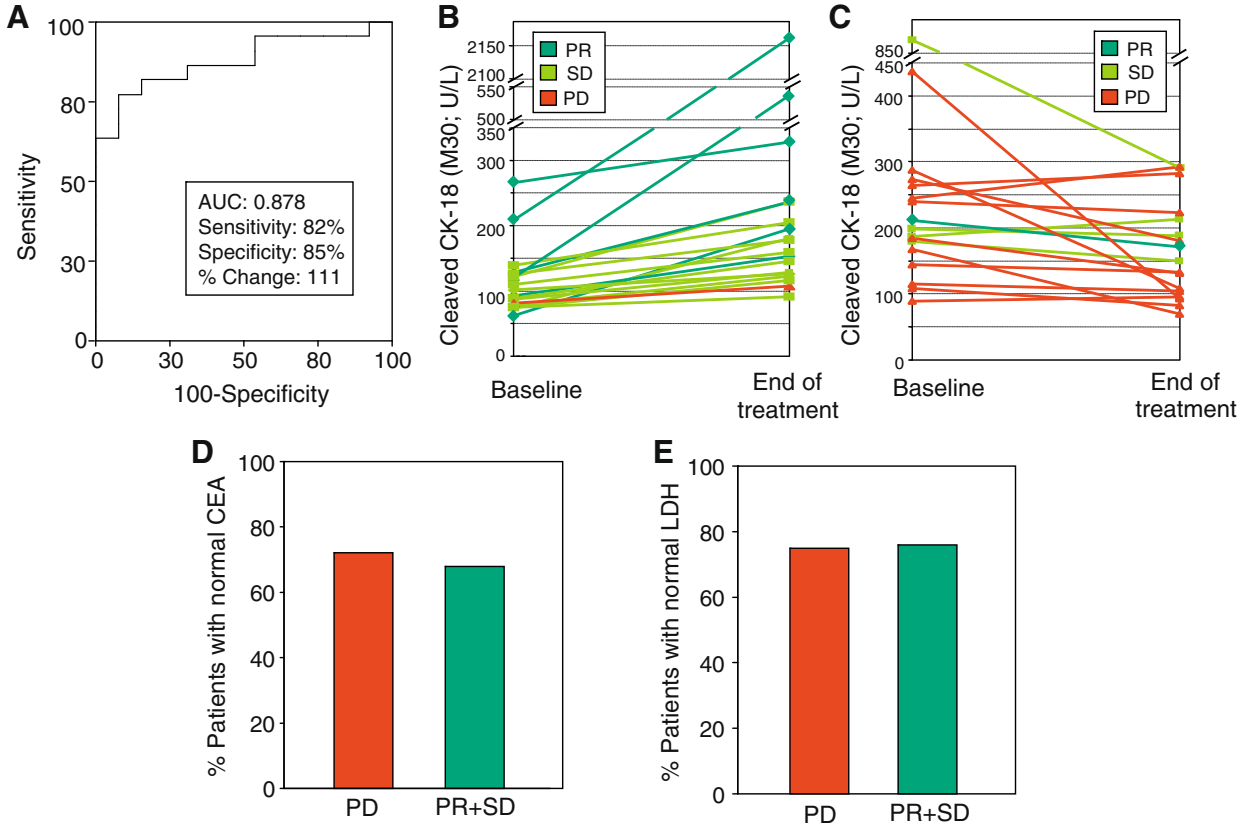

Fig. 5 – Therapy-induced changes of caspase-generated CK-18 fragments and their predictive discriminative value. (A) Predictive discrimination of the relative change of caspase-generated CK-18 fragments during one chemotherapy cycle as determined by receiver-operating characteristic (ROC) plot analysis. The ROC analysis indicates the threshold of percental change of CK-18 fragments for the best-compromise sensitivity/specificity to predict the treatment outcome (AUC, area under the curve and CI, confidence interval). (B, C) Individual therapy-induced changes of M30 levels from baseline (first value) to the end of one cycle of chemotherapy. Patients were grouped into those with a change of M30 levels above (B) or below (C) 111%, as determined by the ROC analysis. (D) Percentage of patients with normal baseline levels of carcinoembryonic antigen (CEA). (E) Percentage of patients with normal levels of lactate dehydrogenase (LDH) during chemotherapy. PR = partial response, SD = stable disease and PD = progressive disease.

The diagnostic importance of measuring soluble products released from apoptotic tumour cells is underlined by the previous studies demonstrating that serum levels of nucleic acids or nucleosomes are increased in cancer patients.[26–28] Furthermore, cytochrome c that is released from mitochondria during apoptosis has been found to increase in sera of patients with haematological malignancies during chemotherapy.[29] Compared to these markers, however, an important advantage of caspase-cleaved CK-18 is that it is specifically expressed by epithelial cells and therefore remains unaffected by chemotherapy-induced apoptosis of non-epithelial cells including bone marrow cells.

In the present study, we found increased caspase-cleaved CK-18 as well as caspase-3 activation in tissue sections of untreated patients with colon carcinoma compared to healthy colon tissues. Several publications have described the relevance of intrinsic apoptosis, as assessed in tissue biopsies, for the clinical outcome of colorectal cancer patients. Whereas some studies reported a better prognosis for patients with high levels of intrinsic apoptosis, others could not find such a correlation or showed the opposite.[30–32] Several reasons might explain these inconsistent findings. For instance, suboptimal surgery can obscure the prognostic value of apoptosis. Furthermore, recurrence-free survival might depend on distant metastasis rather than local control. Another reason for these discrepancies might be the technical limitations of immunohistochemistry as well as the lack of possibilities for reliable quantification of apoptotic cells in tissue sections.

Quantification of cell death in patient sera might circumvent these pitfalls. To this end, we measured caspase-cleaved (M30) and total soluble (M65) CK-18 in sera of patients with gastrointestinal cancers by using specific ELISAs.[14,18] Compared to healthy control individuals, we found significantly elevated caspase-cleaved CK-18 and total soluble CK-18 already in sera of untreated cancer patients. These results are in line with the previous studies demonstrating increased M30 levels in patients with breast, lung or colon cancer.[13,21,22] Although the idea of decreased apoptosis during cancer development is conceptually attractive, in the latter studies M30 levels correlated with tumour stage and progression. An explanation for this observation could be that the continuous cell turnover together with increased apoptosis might select for the survival of tumour cells resistant to apoptosis.

So far there is only a scarce of information about the predictive value of serum cell death markers for the treatment response in cancer patients. At the start of chemotherapy, no significant differences in M30 or M65 baseline levels were found between responding and non-responding patients. We therefore investigated drug-induced changes of M30 and M65 levels in the individual patients in the course of one cycle of anti-cancer treatment. It should be noted that it was not the intention of this exploratory study to compare the effects of different 5-FU/LV-based treatment protocols, but to investigate an association of the cell death markers with the clinical response. In fact, a recent study found no differences in apoptosis induction by different 5-FU/LV-based treatments.[33] In initial experiments, we performed cell death analyses immediately before and after each drug application. However, the most significant drug-induced changes in M30 or M65 values were found at the end of the chemotherapy cycle. One reason for this might be the fact that we have investigated patients with progressed gastrointestinal cancers and aggressive tumour behaviour. In these patients the cumulative effect of several drug applications and the accumulation of caspase-cleaved CK-18 protein complexes, which remain relatively stable in blood,[34,35] might play a role. Furthermore, changes of the expression pattern of pro- and anti-apoptotic molecules might explain the observation that drug-induced effects are most pronounced at the end of the treatment cycle.

Based on this observation, we assessed M30 or M65 levels at the beginning and end of one cycle of chemotherapy. Interestingly, most patients responding to chemotherapy showed an increase of caspase-cleaved CK-18 during the course of chemotherapy. In contrast, patients with progressive disease during treatment generally showed no relevant changes or even a decrease of M30 levels. Statistical analysis confirmed significantly higher changes of M30 levels in patients responding to chemotherapy compared to non-responding patients. Thus, the chemotherapy response is closely associated with increased caspase activation in sera of patients with advanced gastrointestinal cancer.

To further characterise the drug-induced tumour cell death, we calculated the ratio of total soluble CK-18 to caspase-cleaved CK-18. Patients responding to chemotherapy showed a significantly lower M65/M30 ratio at the end of the treatment cycle compared to non-responding patients. Whereas in responding patients the ratio decreased during chemotherapy, non-responders showed an increase of the M65/M30 ratio. These data might indicate that in responding patients the apoptotic cell death predominates and in non-responding patients the non-apoptotic cell death such as necrosis predominates. Such distinct cell death modes could be caused by differences in tumour vascularisation, hypoxia and ATP content between the various prognosis groups. Alternatively, we cannot exclude that the high serum levels of total CK-18 might reflect an increased tumour load and cell turnover in the non-responding patients. It has been recently demonstrated that an increase of total compared to caspase-cleaved CK-18 is associated with tumour progression in colorectal and endometrial cancers.[20,36] Non-apoptotic cell death might be also associated with more aggressive tumour behaviour that could be responsible for treatment failure. A recent study, however, virtually showed increased necrosis in breast cancer patients responding to anthracycline-based chemotherapy.[34] Another study showed that higher baseline CK-18 levels tended to be predictive for worse treatment outcome in gastrointestinal carcinomas.[33] In this study, peak levels of total CK-18 during therapy were associated with tumour response, but peak levels of caspase-cleaved CK-18 did not reach significance. An explanation for these differences compared to our study might be the inclusion of patients with different tumour stages, i.e. metastatic and locally advanced disease, as well as different treatment durations.

Whether the treatment-induced mode of cell death is influenced by different drugs, tumour entities or tumour burden and whether chemotherapy predominantly induces necrosis in apoptosis-resistant tumours remain to be investigated. Interestingly, there is increasing evidence that cell death-induced immune responses might determine the

clinical outcome of chemotherapy.[37] In addition to apoptosis, caspases cleave different cytokine precursors to the active cytokines and thus create an environment essential for an effective anti-cancer immune response.[38] Furthermore, alterations of apoptotic and necrotic cells exert direct immunogenic or tolerogenic effects.[39] Interestingly, recent studies demonstrated that necrotic cell death *in vivo* impaired immune responses, whereas apoptosis was associated with an enhanced tumour-specific immune reaction.[40,41] Among the immunogenic alterations induced in apoptotic cells is the caspase-mediated exposure of calreticulin.[42] Surface-exposed calreticulin promotes the phagocytosis of apoptotic tumour cells by dendritic cells leading to enhanced cytotoxic T-cell responses. Thus, the increased caspase-mediated CK-18 levels in patients with gastrointestinal cancer responding to chemotherapy might eventually reflect a more efficient anti-cancer immune response. Although we are aware that further studies with larger patient cohorts are required to investigate these possibilities and to improve the power of our study, our results indicate that the serum CK-18 levels could serve as valuable predictive marker for the clinical outcome. The future use of cell death biomarkers might improve patient management and allow for the early identification of non-responding patients who might otherwise benefit from alternative anti-cancer therapies.

Conflict of interest statement

None declared.

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