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► **To cite this version:**

C. Ausch, V. Buxhofer-Ausch, U. Olszewski, W. Hinterberger, E. Ogris, et al.. Caspase-cleaved cytokeratin 18 fragment (M30) as marker of postoperative residual tumor load in colon cancer patients. *EJSO - European Journal of Surgical Oncology*, 2009, 35 (11), pp.1164. 10.1016/j.ejso.2009.02.007 . hal-00556281

HAL Id: hal-00556281

<https://hal.science/hal-00556281>

Submitted on 16 Jan 2011

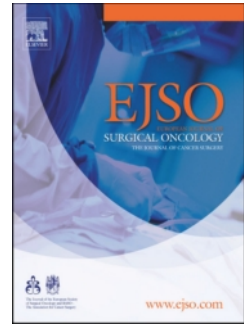
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Accepted Manuscript

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PII: S0748-7983(09)00060-2
DOI: [10.1016/j.ejso.2009.02.007](https://doi.org/10.1016/j.ejso.2009.02.007)
Reference: YEJSO 2795

To appear in: *European Journal of Surgical Oncology*

Received Date: 3 October 2008

Revised Date: 31 January 2009

Accepted Date: 4 February 2009

Please cite this article as: Ausch C, Buxhofer-Ausch V, Olszewski U, Hinterberger W, Ogris E, Schiessel R, Hamilton G. Caspase-cleaved cytokeratin 18 fragment (M30) as marker of postoperative residual tumor load in colon cancer patients, *European Journal of Surgical Oncology* (2009), doi: 10.1016/j.ejso.2009.02.007

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All authors of this submission declare that they have no conflict of interest.

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Abstract

Background: Soluble cytokeratin 18 (CK18; M65) and a caspase-cleaved fragment of CK18 (M30) have been used as biomarkers, corresponding to tumor cell death and apoptosis respectively.

Methods: In the present study, M30 was quantified for the first time in serum samples of colon cancer patients pre- and postoperatively as well as during chemotherapy. Minimal residual disease (MRD) was assessed preoperatively by detection of pan-cytokeratin antibody A45-B/B3-positive cells in bone marrow aspirates.

Results: Out of 46 patients, those with colon tumors of stages I and IV had significantly elevated M30 serum concentrations compared to controls (n = 23). In 31 colon cancer patients, M30 determinations were performed prior to and seven days after tumor surgery. A group of 24 patients exhibited a significant decrease of M30 in response to tumor removal, in contrast to seven patients who revealed either persistent or higher M30 levels postoperatively. The frequency of MRD was not significantly different for patients with decreasing (4/24) and persisting (3/7) M30. However, M30 correlated significantly with the increased number of recurrences within 36 months in the group with persisting M30 (4/7 versus 2/24, p = 0.032; hazard ratio 8.3, p= 0.016). In a group of patients (n = 10) receiving capecitabine/oxaliplatin chemotherapy (CapOx), transient increases in M30 did not correlate with responses.

Conclusion: The data obtained within the present limited pilot study in colon cancer patients demonstrate that perioperative changes of M30 may indicate systemic residual tumor load and increased risk of recurrence warranting further evaluation of this marker of apoptosis in a larger prospective clinical trial.

Key words: colon cancer, cytokeratin 18, apoptosis, caspase, chemotherapy

Introduction

The majority of patients with colorectal cancer present with limited disease, however, despite radical surgery, about 30-50% of these patients develop metastatic disease.¹ The residual tumor load and the extent of pre- and perioperative tumor cell dissemination remains to be determined since appropriate detection systems are not available so far.² A host of diverse tumor markers have been investigated in order to detect residual disease and to aid in prognosis and selection of further therapy.³

Cytokeratins (CKs), proteins belonging to the intermediate filament (IF) family, are particularly useful tools for the surveillance of carcinomas.^{4,5} The three most frequently applied CK markers used in the clinic so far, are tissue polypeptide antigen (TPA) measuring CKs 8, 18, and 19, and the more specific tissue polypeptide-specific antigen (TPS) and cytokeratin fragment 21-1 (CYFRA 21-1) recognizing CK18 and 19, respectively.⁴ Further development of CK-based tumor markers have proceeded to the specific measurement of CK18 and a CK18 fragment, truncated at the N-terminus, from cancerous but not normal cells that is the product of caspase - mediated cleavage during apoptotic cell death.⁶

The levels of such CK18 fragments were significantly different for patients with either lung cancer, benign lung disease or healthy control subjects and predicted survival as well as response to therapy.⁷ Similarly, patients with primary and recurrent breast cancer had higher M30 levels than healthy subjects, and M30 correlated with the number of involved organs and response.^{5,8} In conclusion, assessment of CK18 and the caspase-cleaved CK18 fragment detected by M30 assay has been shown to discriminate patients with cancer from healthy controls, predict survival of the patients and prove efficacy of cytotoxic chemotherapy in different tumor entities.⁵

In the present study, the relevance of a CK18 fragment in colon cancer patients was evaluated using M30 ELISA assays. The different aspects investigated included comparison of preoperative serum concentrations of M30 with clinical parameters, perioperative changes of M30 serum concentrations as well as the influence of CapOx chemotherapy on this CK fragment in a subgroup of the colon cancer patients. Minimal residual disease (MRD) as negative prognostic factor was assessed by detection of cytokeratin-positive tumor cells in bone marrow aspirates.^{9,10}

Patients and methods

Demographic data

A total of 56 patients with colorectal cancer who were treated between January 2002 and December 2004 at the Donaushospital, Vienna and had a follow-up period of more than 3 years. Forty-six patients underwent surgery for primary colorectal carcinoma and ten patients who received palliative chemotherapy for metastasis were included. None of the patients had received chemotherapy and/or radiotherapy prior to surgery. All patients were checked for infections by viral tests, blood count and chemistry, including determination of C-reactive protein. Twenty-three non-tumor patients admitted to the outpatient department for minor complaints served as controls. Bone marrow aspirates of cancer patients were obtained from both upper iliac crests (5 ml each) by needle aspiration. Collected blood was centrifuged 2000 rpm for 10 minutes and stored at -20 °C. For patients with extended disease chemotherapy consisted of either capecitabine/oxaliplatin or irinotecan/irinotecan plus 5-fluorouracil (5-FU) in two cases. Capecitabine was given in a dose of 2 g/m² for d1 – d14, oxaliplatin in a dose of

130 mg/m² on d1 and d22, irinotecan 125 mg/m² weekly, 5-FU, and leucovorin in doses of 1 g/m² on d1 and 500 mg/m² on d1, respectively. Written informed consent was obtained from all patients. The study was approved by the local ethics committee and the institutional review board.

Immunocytochemical analysis and scoring of MRD

Bone marrow aspirates were obtained from both upper iliac crests (5ml each) by needle aspiration immediately prior to the operation under general anesthesia. Mononuclear cells of bone marrow aspirates were separated by Ficoll-Hypaque density gradient centrifugation. Cytospins containing 1x10⁶ cells/slide were fixed in acetone and stained using pan-cytokeratin antibody A45-B/B3 (Micromet, Munich, Germany; final concentration 5 µg/ml; 20 min). Apoptotic tumor cells were detected in deparaffinized tissue sections following treatment of the slides with 100 µg/ml pepsin in 10 mM HCl for 30 min using the M30 Cytodeath antibody (Peviva, Bromma, Sweden; final concentration 0.5 µg/ml, 20 min). All other staining steps, including blocking and washes, were performed using the Idetect-Super-Stain-(alkaline phosphatase)-Fast-Red kit according to the manufacturer's instruction (ID Labs, London, ON, Canada) and mouse monoclonal isotype controls were included. For assessment of MRD at least 2x10⁶ cells per specimen were screened blinded by two pathologists and a minimum of one tumor cell per 2x10⁶ mononuclear cells was regarded as a positive result for A45-B/B3.

M30 and M65 ELISA

From all sera the concentrations of CK-18-Asp396-NE and total CK18 were determined using the M30-Apoptosense[®] and the M65-ELISA assay[®] according to the manufacturer's instruction (Peviva, Bromma, Sweden), respectively. The coefficient of variance for the duplicate measurements of M30/M65 was < 7.5%.

Statistical analysis

Statistical analysis of the control and tumor stage groups was done using ANOVA/Bonferroni correction and Dunnett's test (significance level $p < 0.05$), comparison of the distinct perioperative M30 groups by the Chi Square test, and risk of disease progression by Cox proportional hazards regression (SPSS software, SPSS, Chicago, IL, USA).

Results

Demographic data

For the forty-six patients with newly diagnosed colon cancer mean age was 69 ± 10 yrs (range: 43 – 87 yrs), with 15 female and 31 male patients. 16 patients were UICC stage I, 6 patients stage II, 12 patients stage III, 8 patients stage IV, and 4 patients had local relapses. 11/46 patients (24%) were positive for MRD (50% of the patients with stage IV cancer), exhibiting at least one tumor cell/2x10⁶ mononuclear bone marrow cells. The patient subgroup (n = 31) for perioperative measurements of cytokeratins included the following tumor stages: 13 patients stage I, 3 patients stage II, 6 patients stage III, 5 patients stage IV, and 4 patients with local relapses. Out of this 31 colon cancer patients observed perioperatively, 7 (23%) were positive for MRD. None of the patients revealed signs of infection or inflammation.

Correlation of M30 with tumor stage

M30 was determined in serum samples of 46 colon cancer patients obtained immediately prior to surgery, and mean values were calculated for the different tumor stages (fig. 1; no significant differences among stages). Patients with stages I and IV had significantly higher preoperative values of M30 than the control patients. All other tumor stage groups, including patients with local recurrence, revealed mean M30 values that could not be distinguished from controls. In addition, results of immunohistochemical counting of M30-positive tumor cells (range 1 – 13%) revealed no correlation with either tumor stage due to large variability of the results or serum concentrations of circulating M30 antigen (data not shown). Use of M30 for detection of stage I colon cancers with a cutoff value of 110 U/l M30, corresponding to the mean value for normal controls plus one standard deviation, would result in a sensitivity of 71% and a specificity of 83%. The individual M30/M65 ratios revealed no correlation with tumor stage, grade and other clinical parameters (data not shown).

Effect of tumor surgery on M30 serum concentrations

Serum samples of 31 patients for determination of M30 concentrations were obtained immediately prior to surgery and seven days following intervention. While the whole group responded with a drop of M30 from 154 ± 13 U/l to 103 ± 8 U/l ($-24 \pm 6\%$) to the removal of the tumor, the results showed that in one group of patients (24/31) a reduction of M30 concentrations ($< 95\%$ of the individual M30 serum concentration prior to surgery; 208 ± 34 U/l to 119 ± 19 U/l) was detectable, whereas the second group (7/31) either lacked a decrease or exhibited a significant increase in circulating M30 (87 ± 7 to 122 ± 29 U/l), despite surgery (fig. 2). The two patient groups revealed no significant differences in age (69 ± 8 versus 69 ± 11 years), tumor stage, tumor grade, and other clinical parameters (data not shown), except a significant lower mean M30 serum concentration. Although in these patient groups detection of MRD by assessment of the CK-positive tumor cells in bone marrow aspirates pointed to a lower incidence of dissemination in patients with a reduction of M30 as a consequence of the removal of the tumor tissue, the difference to the group with increasing M30 levels was not statistically significant: 17% (4/24 patients) versus 43% (3/7 patients; $p = 0.14$). Relapses in the group with persisting M30 with 57% (4/7 patients) versus 8% (2/24 patients) were significantly elevated ($p = 0.032$), yielding a hazard ratio of 8.3 ($p = 0.016$). Only two of the relapsing patients proved to be MRD-positive. In the group characterized by decreasing perioperative M30 the duration to relapse was 5 and 21 months, and in the group with increasing M30 four patients relapsed after 7, 6, 11, and 14 months, respectively. Of the former two patients one had tumor recurrence in the beginning and the other showed a late relapse after 21 month. Time to progression was 69.4 ± 4.5 month (95% confidence interval: 60.7 – 78.1 month) for the group with decreased perioperative M30 serum concentrations and 29.7 ± 10.3 month (95% confidence interval: 9.5 - 50 month) for the group with persisting M30 antigen.

M30 and chemotherapy

To assess the effects of chemotherapy on tumors and possible release of M30, serum samples were drawn from patients with metastatic colon cancer prior to initiation of chemotherapy ($t = 0$ h) and 24, 48, 72, 96 hrs thereafter (fig. 3). Patients received CapOx, with exception of two patients exhibiting progressive disease who were treated with irinotecan and 5-FU/irinotecan, respectively. In this small set of patients one complete and one partial response, five cases of stable disease and three cases of progressive disease were observed. As shown in fig. 4,

patients with stable disease revealed a transient increase of M30 48 hrs following initiation of chemotherapy, patients with progressive disease a peak value of M30 24 hrs after start of therapy, and the two patients with responses lacked a significant increase in M30.

Discussion

In the present study we investigated for the first time the significance of serum concentrations of M30, the caspase-cleaved fragment of CK18 (M65), in colon cancer patients using ELISA assays.¹¹ CK18 and its fragment are released by tumor cells during necrosis and apoptosis, respectively, and are expected to correlate with tumor mass, tumor stage and response to chemotherapy as demonstrated in breast, colorectal, endometrial, lung and prostate cancers, among others.^{5,12}

M30 and tumor stage

Preoperative values of M30 were significantly increased for the colon cancer patients bearing stage I and IV tumors compared to control patients. These patients revealed no signs of infection or inflammation that were reported to result in increased release of cytokeratin 18 fragments by normal epithelial cells.^{4,5} According to published data immunohistochemistry using the M30 antibody demonstrated an increased fraction of apoptotic cells following the order of normal colorectal mucosa, adenomas, and carcinomas, and tumors at early stages exhibit increased apoptotic cell death.^{13,14} Stage IV tumors may be characterized by increased cell death due to insufficient vascular supply. Patients with UICC tumor stages II–III exhibited M30 serum concentrations that were not statistically significant from control patients. Stabilization of tumor cells and decrease of apoptotic cell death have been reported for higher tumor stages in breast cancer.¹⁵ According to our data there is no clear relationship between the number of M30 positive tumor cells and M30 serum concentrations.

Perioperative concentrations of M30

For a group of 31 colon cancer patients, preoperative determinations of M30 were compared to measurements obtained one week after surgical removal of the tumor. One subgroup responded to surgery with a decrease of M30 to control levels, whereas the other subgroup failed to show any reduction in circulating M30 and even exhibited higher concentrations of this antigen. Recombinant CK18 was reported to have a half-life of 2.3 days in normal human plasma at 37 °C and therefore removal of the source of soluble CK18 is expected to be followed by a rapid drop of its concentration in the circulation during one week.¹¹ Actually, a rapid reduction of CK19 fragments (CYFRA 21-1) within one day following surgical intervention has been described for lung cancer patients.¹⁶ Therefore, remaining disseminated tumor cells seem most likely to be responsible for the persistent production and release of M30 following radical surgery. Patients with persisting M30 elevations showed no signs of infection or inflammation and these increased circulating M30 antigen is not likely due to the surgical intervention, since even trauma patients exhibited values within the normal range.¹⁷

Prognostic relevance of perioperative M30 measurements

MRD may be detected by analysis of bone marrow aspirates using epithelial-specific markers for detection of cancer cells.⁹ Since bone metastasis is less frequent in colon cancer, such tumor cells occurring in bone marrow may be a sign of spread to other organ sites.¹⁸

However, the two colon cancer groups with divergent courses of serum M30 lacked a significant difference in their frequency of MRD-positive bone marrow aspirates. Of the six patients exhibiting tumor recurrence only two patients were positive for pan-CK-expressing bone marrow cells. In good agreement with the literature this data lack to support a prognostic significance of MRD in colorectal cancer.¹⁹ In contrast, the frequency of early tumor recurrences was significantly higher in the patient group exhibiting persisting elevation of serum M30 following tumor surgery. Interestingly, these patients had significant lower absolute M30 serum levels than the patients showing perioperative decreases of M30 and a lower number of relapses. Again, low expression of CK18 may indicate a more aggressive cancer phenotype, as described for breast cancer.¹⁵ In a small set of these colon cancer patients, responding patients failed to exhibit a significant release of M30 in response to CapOx therapy and patients with stable disease or progression showed a transient increase of M30 after 24–48 hrs in response to chemotherapy indicating a lack of prognostic significance of this elevation of M30 for these colon cancer patients. The results indicate that necrosis may be the predominant type of cell death under these conditions, as observed in response to chemotherapy in other tumors.¹²

Conclusion

In conclusion, serum concentrations of M30 are elevated in low and advanced stages of colon cancer and the difference of preoperative and postoperative serum concentrations of this antigen seems to represent an interesting marker of residual tumor load and early tumor recurrences. However, no specific changes of M30 were found within the first four days following initiation of CapOx chemotherapy in patients with metastatic disease. The results of this pilot study indicate that M30 may warrant consideration as an extremely early prognostic marker of tumor dissemination and progression to identify patients at increased risk of tumor recurrence, depending on confirmation of these, preliminary results in a much larger series of colon cancer patients in a prospective clinical trial.

Conflict of interest and acknowledgements. The authors have no conflict of interest. This work was supported by the *Fonds for Innovative Cancer Research of the City of Vienna* and the *Society for Research on Biology and Treatment of Tumor Diseases*.

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Legend to the figures

Figure 1. Mean preoperative serum concentrations of M30 of controls and colon cancer patients for the different UICC tumor grades and (mean \pm SEM). Mean values of groups that are significantly different from control group are indicated by an asterisk ($p < 0.05$).

Figure 2. Comparison of preoperative serum concentrations of M30 (d0) with postoperative values (d7) in 31 colon cancer patients exhibiting either perioperative reduction ($< 95\%$) or increases in relation to preoperative concentrations of this antigen.

Figure 3. Time course of M30 serum concentrations (mean \pm SD) in groups of patients with either stable (SD) or progressive (PROGR) disease and partial responders (PR) during chemotherapy.

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