Serum Mannan-Binding Lectin-Associated Serine Protease 2 Levels in Colorectal Cancer: Relation to Recurrence and Mortality

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ABSTRACT

Purpose: **Mannan-binding lectin-associated serine protease 2 (MASP-2) is a plasma protein involved in inflammatory processes. MASP-2 circulates in complex with the protein mannan-binding lectin (MBL) or ficolins, and is activated to recruit the complement system when MBL binds to its targets. The level of MASP-2 is genetically determined, and the aim of the present study was to evaluate the effect of MASP-2 levels on postoperative infection, recurrence and survival.**

Experimental Design: **MASP-2 concentrations were determined in serum from 605 patients collected before elective resection for primary colorectal cancer. The primary end points were postoperative infection, time to any recurrence, and time to death. The median time of follow-up was 7.9 years.**

Results: **MASP-2 levels were not correlated to postoperative infections ($P = 0.49$). High MASP-2 levels significantly correlated with recurrent cancer disease [$P = 0.03$; hazard ratio (HR) = 1.4; 95% confidence interval (CI), 1.0-2.0] and with poor survival ($P = 0.0005$; HR = 1.4; 95% CI, 1.2-1.7). Multivariate statistical analysis, including age, gender, Dukes' stage of disease, tumor localization, and postoperative pneumonia, showed that the MASP-2 level had an independent prognostic value in the patients ($P = 0.0001$; HR = 1.5; 95% CI, 1.2-1.8).**

Conclusion: **In the cohort of patients with colorectal cancer investigated, MASP-2 concentration in serum proved to be an independent prognostic marker with high MASP-2 levels predicting recurrence and poor survival. Postoperative infection could not be shown to be associated with MASP-2 levels.**

INTRODUCTION

Colorectal cancer is the second leading cause of death by malignant disease. Approximately half of patients with colorectal cancer considered curatively operated will develop recurrent disease within 5 years. The risk of recurrence after intended curative surgical intervention is multifactorial and seems to include postoperative infectious complications because these lead to increased risk of recurrence and poor long-term survival (1–4). This finding is supported by reports on other solid tumors (5–8). Despite antibiotic prophylaxis at surgical resection of colorectal cancer, 5% to 30% of the patients develop postoperative bacterial infectious complications. Impaired pre- and postoperative immune competence, including impaired complement activation pathways, have been implied (9–13). More detailed knowledge of the immune function in colorectal cancer may offer opportunities to improve the disease course. Furthermore, conventional staging of colorectal cancer does not account for the marked variability in outcome within each stage, and identification of molecular prognostic markers for evaluating the individual patient is important for the clinical management of the patient. Thus, there is a need for biomarkers to characterize disease type, status, progression, and response to therapy in colorectal cancer.

The mannan-binding lectin (MBL) pathway of complement activation is an innate immune defense mechanism that responds directly to pathogens. MBL circulates in the blood in complex with MBL-associated serine proteases (MASP-1, -2, and -3). MASP-2 is the protease responsible for activating the complement cascade, whereas the roles of MASP-1 and MASP-3 are unknown. The MBL pathway of complement activation is initiated upon binding of MBL to the carbohydrates present on the surface of microorganisms. This causes MASP-2–mediated activation of C4 and C2 to generate C3 convertase (C4bC2b), leading to neutralization of the pathogen (14–17). Interestingly, MBL has also been suggested to have a cytotoxic effect on colon adenocarcinoma cells *in vitro* (18, 19) and *in vivo* (20). The MBL concentration in serum and the activity of the MBL pathway is generally increased in patients with colorectal cancer (21). Insufficient levels of MBL and low activity of the MBL/MASP complexes, are associated with an increased risk of postoperative pneumonia and possibly also with other postoperative infectious complications in patients with colorectal cancer (22, 23). In a previous study, low MBL

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levels could not be associated with recurrent cancer disease or survival (23). MASPs not only function in complex with MBL but also mediate the biological activity of two other proteins, L-ficolin and H-ficolin (24, 25). With a recently developed assay (26), it is now possible to determine the total level of MASP-2 in serum. The aim of the present study was to determine the MASP-2 levels in patients with colorectal cancer and to evaluate the possibility of an association between the MASP-2 levels and postoperative infection, recurrence, and survival.

PATIENTS AND METHODS

Patients scheduled for elective resection of primary colorectal cancer were included in the study. All 605 patients were without clinical signs of infectious disease, and none had been treated with antibiotics or immunosuppressive agents such as systemic steroids, antiviral drugs, or cytotoxic agents within 2 weeks before surgery. Patients with severe concurrent illness, such as leukemia, autoimmune disease, or HIV infection were not included. None of the patients received pre- and/or postoperative chemo- and/or radiotherapy. None of the patients presented with bowel obstruction or perforation, because only patients undergoing elective surgery were included. Blood from the patients was collected just before skin incision.

In total, 238 women and 367 men with histologically verified primary colorectal cancer were included between April 1991 and August 1993. The median age at the time of tumor resection was 69 years (range, 33-91 years) and the patients were observed for a median of 7.9 years (range, 6.5-9.1 years). Patient data included localization of the tumor to colon or rectum and disease stage according to Dukes' classification with addition of a D group identifying patients with distant metastasis. Postoperative infectious complications were recorded as described at the end of this section.

In addition, blood was collected at the blood bank from 150 blood donors, 55 to 65 years of age, and serum was prepared. Health registrations of the blood donors in Denmark are taken before every blood donation; thus, this group is well characterized. In particular, the donors cannot give blood if they have ongoing infections. The peripheral blood samples from the patients and donors were collected in endotoxin-free silicon tubes (Becton Dickinson, Mountain View, CA) without additives. The samples were kept at room temperature for 1 hour after the collection, centrifuged (2,000 × *g*, 4°C, 10 minutes), and the serum was removed and stored at −80°C until analyzed.

The investigation was carried out according to the Helsinki declaration. All patients as well as donors gave informed consent to participate, and the study was approved by the Regional Ethics Committees. The patients were part of a previously described prospective, double-blind, placebo-controlled, clinical study of the efficacy of the histamine-2 receptor antagonist ranitidine on long-term survival after resection of primary colorectal cancer (27).

A sandwich-type, time-resolved immunofluorometric assay using a combination of two monoclonal anti–MASP-2 antibodies was used for quantifying MASP-2 in serum. The assay has been described in detail elsewhere (26). In brief, serum is incubated in microtiter wells coated with anti–MASP-2 antibody (8B5). Subsequently, europium-labeled anti–MASP-2 antibody (6G12) is added and, after washing, quantified by fluorometry. The serum dilution routinely applied (1/75) allows for the detection of between 10 ng and 10 μg MASP-2/mL serum. The assay measures total MASP-2 because it is done in a high-salt, EDTA-containing buffer, conditions dissociating all MASP from MBL and ficolin complexes.

End points. Survival was considered the primary end point. Furthermore, we examined the development of postoperative infection and any recurrence (either distant metastasis or local recurrence).

The postoperative infectious complications were classified as follows. (*a*) Wound infection was defined as the presence of pus, either discharged spontaneously or requiring drainage. Wound infection included a subgroup of patients with perineal infection after abdominoperineal resection of the rectum. (*b*) Intra-abdominal abscess was verified by either surgical drainage or by ultrasonographically guided aspiration of pus. (*c*) Anastomotic leakage was defined as radiological verified fistula to bowel anastomosis or it was diagnosed by relaparatomy. (*d*) Pneumonia was defined by fever above 38.5°C, a positive X-ray, and the requirement of antibiotic treatment. (*e*) Septicemia was defined by clinical symptoms combined with a positive blood culture. Nonsymptomatic or minor urinary tract infection was not recorded, and therefore included only if complicated by septicemia. Registration of these complications was carried out on a daily basis. We investigated infections in general, and specifically, pneumonia.

After discharge, the patients were examined in the outpatient clinic every 3 months for up to 5 years, with registration of date of local recurrent disease or metastasis, and thereafter when required. Overall survival was surveyed through the National Patient Registry.

Statistics. The SAS software package (version 8.2; SAS Institute, Cary, NC) was used for data management and statistical calculations. The MASP-2 concentrations are presented by the quartiles (median) and range. Hypothesis tests on location were done using the Wilcoxon rank sum test. The associations between the variables were estimated by Spearman rank correlation. Estimates of overall survival were done using the Kaplan-Meier method. The Cox proportional hazards model was used for multivariate analysis of recurrence and survival. The covariates included in the model are disease stage, localization of the tumor, age, gender, infectious complications, perioperative blood transfusion, and treatment as well as the marker included in this study. The marker MASP-2 was entered into the model as the log of the actual level. All P values <5% were considered significant.

RESULTS

Patient characteristics including tumor localization, Dukes' stage, and MASP-2 concentrations are shown in Table 1.

The preoperative MASP-2 levels were significantly increased (P = 0.0002) among patients with colorectal cancer (median, 415 ng/mL; interquartile range, 312-543 ng/mL; total

Table 1 Basic patient characteristics (gender, median observation time, Dukes' stage, tumor localization) and MASP-2 concentration in serum in the patients (N = 605)

		MASP-2 concentration (ng/mL)		
Patients, n (%)		Median	Interquartile range (range)	P
Gender				
Female	238 (39)	409	308-502 (40-1,559)	0.10
Male	367 (61)	420	312-564 (37-2,345)	
Dukes' stage				
A	63 (10)	433	299-629 (127-1,112)	0.08
B	229 (38)	390	299-500 (37-1,599)	
C	179 (30)	420	318-526 (62-1,570)	
D	134 (22)	438	338-593 (142-2,345)	
Tumor localization				
Colon	341 (56)	412	299-545 (37-1,559)	0.21
Rectum	264 (44)	430	331-542 (104-2,345)	

range, 37-2,345 ng/mL) as compared with the levels among healthy blood donors (median, 368 ng/mL; interquartile range, 218-481 ng/mL; range, 18-891 ng/mL).

The MASP-2 levels could not be shown to be dependent on the Dukes' stage of disease (P = 0.08), gender (P = 0.10), or tumor localization in rectum or colon, respectively (P = 0.21). The MASP-2 levels were weakly associated to age of the patients (Spearman's ρ:r = −0.16, $P < 0.0001$). No correlation was found when comparing the MASP-2 levels to MBL levels (determined in a previous study; ref. 23): Spearman's ρ:r = 0.07 (95% CI, −0.01 to 0.15; P = 0.09).

MASP-2 and Postoperative Infectious Complications. The MASP-2 levels were not associated with postoperative infections (n = 156) as shown in Table 2. The number of the various infectious complications is low with the exception of pneumonia (n = 87), and therefore the power to detect significant differences is limited.

MASP-2 and Recurrent Cancer Disease. Statistical analysis of time to any recurrence showed a significant association between the preoperative MASP-2 levels and recurrent disease (Fig. 1), with high MASP-2 levels associated with increased risk of recurrent disease (P = 0.02, trend test). This analysis was restricted to curatively resected patients. Applying multivariate analysis with the inclusion of age, gender, Dukes' stage of disease, tumor localization, perioperative transfusion of blood, and postoperative pneumonia in the curatively treated patients (Dukes' stage A-C, n = 451) showed that the preoperative MASP-2 level had statistically independent prognostic value with respect to recurrence (P = 0.03). The results of the multivariate analysis are shown in Table 3.

Table 2 MASP-2 levels in postoperative infectious complications

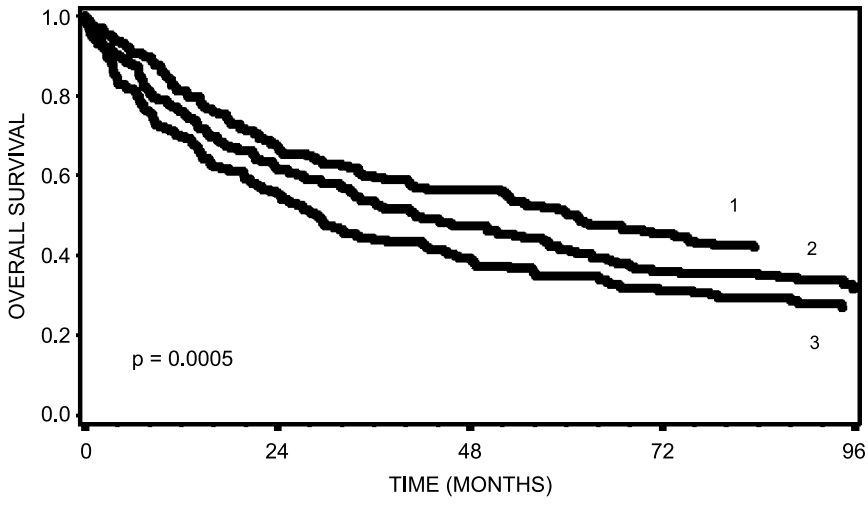
Infection (N = 156)	n	Median (range)	P
Wound infection, total	55	437 (103-1,570)	0.85
Perineal infection*	22	473 (104-1,570)	0.29
Intra-abdominal abscess	21	423 (103-1,056)	0.26
Anastomotic leakage	37	472 (104-1,087)	0.18
Pneumonia	87	432 (130-1,202)	0.49
Septicaemia	20	415 (143-2,345)	0.84

NOTE. In some patients, more than one infectious complication was observed.

*Perineal infection is a risk only in patients undergoing abdominoperineal surgery (n = 95).

MASP-2 and Overall Survival. Among the patients, high levels of MASP-2 (log transformed) corresponded to poor survival (P = 0.0005). This association is illustrated by a Kaplan-Meier plot in Fig. 2 showing the survival by MASP-2 levels grouped by the tertiles.

The MASP-2 levels were shown to have a statistically independent prognostic value with respect to survival ($P < 0.0001$, Table 4) when applying multivariate statistical analysis including age, gender, Dukes' stage, perioperative blood transfusion, tumor localization, and postoperative pneumonia. When including the treatment with ranitidine or placebo in multivariate



DEATHS	PATIENTS AT RISK				
117	202	136	114	92	T 1
137	205	127	97	74	T 2
143	198	110	78	62	T 3

Fig. 1 MASP-2 serum levels and recurrent cancer disease. Kaplan-Meier plot illustrating the recurrence of cancer in the curatively treated patients (n = 451) in relation to preoperative MASP-2 levels (log transformed) grouped by tertiles (the first tertile is 341 ng/mL, and the second tertile is 473 ng/mL). The number of patients with recurrence and the number of patients at risk at 0, 24, and 48 months in each stratum are shown below the graph. The P value for trend test is presented.

Table 3 Results of multivariate analysis estimating recurrent cancer disease in patients considered curatively operated for the cancer (Dukes' stage A-C)

Covariate	*P*	HR (95% CI)
Age (y)	0.40	1.01 (0.99-1.02)
Gender (male vs female)	0.80	1.04 (0.8-1.4)
Dukes' stage B vs A	0.004	3.0 (1.4-6.4)
Dukes' stage C vs A	<0.0001	8.1 (3.9-16.9)
Rectum vs colon	<0.0001	2.0 (1.4-2.8)
Transfusion	0.08	1.4 (1.0-1.9)
Pneumonia	0.11	1.4 (1.0-1.9)
MASP-2 (log transformed)	0.03	1.4 (1.0-2.0)

NOTE. The Cox proportional hazards model is used. $N = 451$, 158 recurrences.

analysis of overall survival ($n = 605$), no statistical difference between the patients treated with ranitidine and placebo, respectively, was detected ($P = 0.29$). An interaction between ranitidine treatment and high MASP-2 levels (dichotomizing by the median) was found in patients with rectal cancer [$P = 0.04$; HR = 1.5; 95% CI, 1.0-2.2].

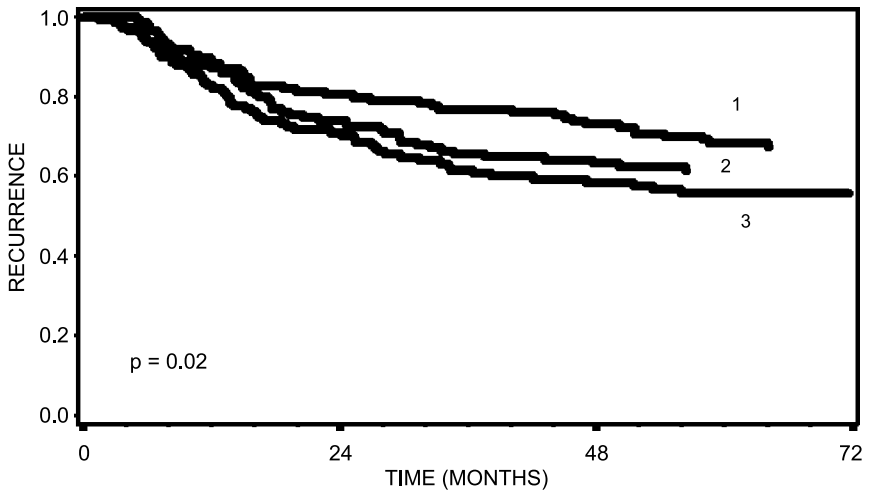
DISCUSSION

In this study, preoperative MASP-2 levels were found to be elevated in serum from patients with colon or rectal cancer compared with those in healthy controls. There was no association between the serum levels of MASP-2 and the frequency of postoperative infections including pneumonia, but with increasing MASP-2 levels, recurrent cancer disease was more frequent and the survival poorer. This highly significant effect of MASP-2 on prognosis was observed in univariate analysis of all 605 patients illustrated in Fig. 2.

Clinical studies suggest that insufficient MBL pathway activity due to MBL deficiency leads to an increased risk of postoperative infectious complications, mainly pneumonia, which is associated with poorer survival (22, 23). In the present study, we did not detect any association between the isolated MASP-2 levels and pneumonia or other infections; that is, MASP-2 does not as a single factor influence the risk of developing postoperative infections in patients with colorectal cancer.

These patients were recruited before the implementation of the total mesorectal excision approach to rectal cancer resections, and this may explain the finding that the prognosis with respect to recurrent cancer and survival is poorer in patients with rectal cancer. Hence, the surgical access to the rectum and perirectal structures was more difficult, resulting in a higher frequency of intra- and postoperative complications including bacterial infections, anastomotic leakage, and need for transfusion (3).

When adjusting for age, gender, Dukes' stage, tumor localization, postoperative pneumonia, and perioperative blood transfusion in multivariate analysis (Tables 3 and 4), a high MASP-2 level was still an independent prognostic parameter, predictive of recurrence and poor survival in rectal as well as colon cancer. Although the biological implication of these findings is still to be investigated, the association points to a role of MASP-2 as a new prognostic biomarker in colorectal cancer. Furthermore, MASP-2 is, unlike other biomarkers of colorectal cancer, a well-described part of the innate immune system (28, 15, 17). Whether the biological function of MASP-2 plays a role in relation to the prognosis of colorectal cancer is unknown. Presently, only a few studies have focused on the possible role of the components of the MBL pathway for colorectal cancer, and determining MASP-2 levels in serum has only recently become an option. The MBL pathway activity and the serum concentration of MBL has been shown to be increased in patients with colorectal cancer compared with healthy controls (21), and now, in addition, we find increased MASP-2 levels in the patients with colorectal cancer. The serum levels of MASP-2 and MBL were not correlated, although these substances



RECURRENCES	PATIENTS AT RISK			
45	151	109	95	T 1
52	152	99	79	T 2
61	148	93	72	T 3

Fig. 2 MASP-2 serum levels and survival. Kaplan-Meier plot illustrating the survival of the patients ($n = 605$) in relation to preoperative MASP-2 levels (log transformed) grouped by tertiles (the first tertile is 347 ng/mL, and the second tertile is 491 ng/mL). The number of deaths and the number of patients at risk at 0, 24, and 48 months in each strata are shown below the graph. The *P* value for trend test is presented.

circulate as a complex in the blood and together act to neutralize pathogens via the MBL complement-activating pathway. Because the assay used in this study detects total MASP-2 (i.e., MASP-2 in complex with MBL as well as with ficolins), the lack of correlation between MBL and MASP-2 levels indicates a substantial fraction of MASP-2 being complexed with ficolins, as suggested in some studies (24). Until now, focus has been set on the complement-activating function of the MBL pathway, because this is the first discovered and best-characterized function. The biological role of MASP-2 complexed with ficolins is poorly illuminated. L-ficolin does react with some pathogenic bacteria, but H-ficolin has thus far only been found to react with a common nonpathogenic bacterium, *Aerococcus viridans* (24). It is possible that MBL/MASP and/or ficolin/MASP complexes may subserve functions outside the innate immune defense. It could be hypothesized that the increased MASP-2 concentrations in patients with colorectal cancer could be due to an inflammatory response, as is the case for other parameters. Hence, a preoperative increase in the acute phase protein, C-reactive protein, is known to be associated with poor prognosis with respect to cancer recurrence and survival (29, 30). In a preliminary study, the preoperative MBL levels and MBL/MASP-initiated complement activation was compared with the preoperative C-reactive protein levels and found not to be correlated to the increased C-reactive protein levels (23). A recent article suggested that prolonged activation of signaling pathways through inflammation may play a role in cancer development and growth (31).The increased MASP-2 levels in patients with colorectal cancer could be hypothesized to play such a role in this condition, which is also in concordance with the finding of an increasingly poorer prognosis with higher levels of MASP-2. Because MASP-2 is a protease, a possible function of the free form of the molecule could be facilitation of tumor invasion as shown for other proteases (32, 33). Alternatively, MASP-2 may be part of the molecular pathways that allow communication between abnormally growing cancer cells and inflammatory cells found in the tumor microenvironment, which has been described in another recent article (34). A second hypothesis is that the low levels of MASP-2 associated with better prognosis in terms of recurrent cancer and survival reflects a high consumption of MASP-2, which may act as cancer prevention, for example, by inhibiting tumor growth. Reversing the causality suggests a third hypothesis that the most aggressive tumors stimulate MASP-2 synthesis.

Finally, the association between high MASP-2 levels and poor prognosis, found in the present study, may be a common result of a shared underlying mechanism.

In the present study, eight patients (seven of these >72 years of age) were MASP-2 deficient when using a threshold of 100 ng/mL (35). Only one of these patients developed recurrent cancer disease, and only three died during follow-up, despite ages >72 years of seven of the patients at diagnosis. None of these patients developed postoperative infectious complications. These numbers are too small to draw conclusions about the role of MASP-2 deficiency.

The patient cohort is from the RANX05 trial (27) in which the patients were randomized to treatment with ranitidine or placebo to compare the effect of ranitidine on the survival in patients electively resected for colorectal cancer. In addition, this study included the analysis of the effect of prognostic factors on treatment and postoperative complications. Hence, we tested for the influence of the treatment with ranitidine or placebo on survival. The ranitidine treatment was not found to be associated with overall survival of the patients. However, we found an interaction between ranitidine treatment and high MASP-2 levels in the patients with rectal cancer, but not in patients with colon cancer. There are no obvious biological theses for this difference in effect dependent on localization of the tumor. Perioperative transfusion of blood is suggested to negatively influence the survival in patients with colorectal cancer (3, 36). In the present study, transfusion was not independently predictive of survival.

Table 4 Results of multivariate analysis, estimating overall survival using the Cox proportional hazards model ($N = 605$, 397 deaths)

Covariate	*P*	HR (95% CI)
Age (y)	<0.0001	1.03 (1.02-1.04)
Gender (male vs female)	0.02	1.3 (1.0-1.6)
Dukes' stage B vs A	0.005	2.0 (1.2-3.2)
Dukes' stage C vs A	<0.0001	4.2 (2.6-6.8)
Dukes' stage D vs A	<0.0001	20.7 (12.7-33.9)
Rectum vs colon	0.07	1.2 (1.0-1.5)
Transfusion	0.56	1.1 (0.9-1.3)
Pneumonia	0.02	1.4 (1.0-1.8)
MASP-2 concentration	<0.0001	1.5 (1.2-1.9)

In conclusion, an association between MASP-2 levels and postoperative infectious complications could not be shown. The study showed that high levels of preoperative MASP-2 were associated with the risk of recurrent disease and poorer survival in patients with colorectal cancer. Thus, MASP-2 holds significant prognostic information suggesting a role for this immunologic parameter as a biomarker in colorectal cancer disease.

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