Immunohistochemically Detected High Expression of Matrix Metalloproteinase-2 as Predictor of Poor Prognosis in Duke's B Colon Cancer

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Aim To demonstrate immunohistochemical expression of matrix metalloproteinase-2 (MMP-2) protein in Duke's B colon cancer and determine its correlation with age, sex, grade, presence of vascular invasion, and patients' overall survival.

Method The study took place from January 1995 to December 1997. We determined the expression of MMP-2 in 152 formalin-fixed, paraffin embedded specimens of Duke's B colon carcinomas by immunohistochemical analysis using MMP-2 monoclonal antibody. Immunohistochemical expresssion was scored semiquantitatively. Carcinomas were graded as low or high grade. Survival time was analyzed with Kaplan-Meier method, and the log-rank test was used to assess the differences between groups. Cox proportional hazard regression model was used for multivariate survival analysis.

Result Univariate analysis showed that positive staining for MMP-2, high histological grade, vascular invasion, male sex, and age >60 years were associated with shorter survival in patients with Duke's B colon cancer (P range from 0.023 to <0.001). Multivariate analysis showed that only MMP-2 overexpression ($P < 0.001$; hazard ratio [HR] = 3.64) and vascular invasion ($P < 0.001$; HR = 4.27) were associated with shorter overall survival.

Conclusion Expression of MMP-2 is an important independent indicator of shorter survival in patients with Duke's B colon cancer and should be taken into consideration in decision-making on the use of adjuvant systemic therapy in patients with Duke's B colon cancer.

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Colorectal carcinoma (CRC) is the third leading cause of cancer-related mortality in developed countries (1). Despite improvements in surgical and adjuvant chemotherapy treatment, mortality from CRC in Western countries remains high, with metastatic spread to the liver occurring in about 50% of patients (2). Although staging remains the most widely used prognostic indicator for CRC, increasing evidence suggests that it is not sufficient for predicting the clinical outcome of these patients (2). This applies especially to patients with intermediate stage diseases (Duke's B, T3-4N0M0), since clinical management for them has yet to be standardized (2). Clinical staging may be supplemented by the use of biological prognostic markers for invasion and metastasis. They may provide important information needed for the implementation of various novel therapeutic strategies for controlling disease progression and tumor cell dissemination (2).

Tumor cell invasion and metastasis are multi-step phenomena, involving the proteolytic degradation of the basement membrane and the extracellular matrix, altered cell adhesion, and physical movement of tumor cells. It was shown that degradation of basement membrane and extracellular matrix play a crucial role in tumor invasion and metastasis (3).

Tumor cells secrete proteolytic enzymes or induce host cells to secrete proteases. Extracellular matrix degradation by proteases takes place not only in local invasion, but also in several stages of metastatic cascade, including angiogenesis, intravasation, and extravasation. The proteases involved in extracellular matrix degradation in tumor invasion and metastasis are subdivided into four classes as follows: serine, cysteine, aspartic, and matrix metalloproteinases (MMPs). MMP-2 is responsible for degradation of collagen type IV, which is the major structural protein in the basement membrane. Therefore, activation of MMP-2 is a crucial step in triggering the cascade of tumor invasion and metastasis (4).

The prognostic significance of MMP-2 overexpression in humans has been shown in breast cancer (5), head and neck tumors (6), and ovarian carcinomas (7).

The aim of this study was to determine the expression of MMP-2 using immunohistochemical methods in a subpopulation of patients with Duke's B colon cancer and examine its relationship with clinicopathological parameters and patient survival.

Patients and methods

Patients

This study included histological samples from 152 patients diagnosed with Duke's B colon cancer at the Department of Pathology, Split University Hospital, Split, Croatia, from January 1995 to December 1997.

Clinical data were collected from the Department of Oncology, Split University Hospital. Survival time of the patients was calculated as the interval from the date of diagnosis to the date of the last clinical control or death from the CRC-related causes until December 31, 2006.

We determined conventional histopathological prognostic parameters of CRC, such as tumor grade, depth of invasion, and vascular invasion in hematoxylin-eosin sections of the specimens. Tumors were staged according the Duke's staging system (8) and stratified into low and high grade tumors, as recommended by a multidisciplinary colorectal working group of a Consensus Conference, sponsored by the College of American Pathologists (9). According to this system, stratification is based solely on the proportion of gland formation by the tumor – low grade with <50% gland formation and high grade with ≥50% gland formation.

Immunohistochemical staining

We performed immunohistochemical staining on 5 μm-thick sections of the most representative paraffin blocks from each tumor.

Slides were dried overnight at 60°C and deparaffinized in xylene. Subsequently, they were rehydrated through graded alcohols into water. Heat-induced epitope retrieval was achieved by boiling sections in an EDTA buffer, pH 8.9, in a microwave oven at 1000 W for 20 minutes (4 times per 5 minutes each). After boiling, sections were left to cool at room temperature for 20 minutes, rinsed thoroughly with water, and placed in TRIS-buffered saline (TBS) for 5 minutes. Endogenous peroxidase was blocked with Peroxidase Block solution (provided in the EnVision kit, DacoCytomation, Glostrup, Denmark) for 5 minutes, and slides were rinsed with TBS. Sections were incubated for 30 minutes with primary mouse monoclonal anti-human MMP-2 antibody (clone MAB 902, dilution 1:30, R&D systems, Minneapolis, MN, USA).

Positive signal for MMP-2 was located in the cytoplasm of tumor cells and the stainability was semiquantitatively estimated on the basis of percentage of positive stained tumor cells. The stainability was scored as positive if ≥10% stained cells were found (7,10). We used slides of human placenta as positive control for MMP-2 immunohistochemical staining, as recommended by the manufacturer (R&D systems). The same sections were processed without primary antibodies as negative control.

Statistical analysis

χ^2 test was used to examine the association between increased MMP-2 expression and various clinicopathological characteristics. The Kaplan-Meier method was used to estimate survival as a function of time, and survival differences were analyzed with log-rank test. Cox proportional hazard regression model was used to simultaneously examine all factors found to be prognostic of survival in univariate analysis. Analyses were performed with Statistical Package for Social Sciences, version 9.0 (SPSS Inc., Chicago, IL, USA).

Results

The mean age of patients was 62 ± 8.8 years (range, 39-79). There were 57 (37.5%) women and 95 (62.5%) men.

During the follow-up period, 86 (56.6%) patients died – 72 (46.1%) from distant metastasis and 14 (10.9%) from reasons unrelated to CRC. Median survival time was 60.5 months (range, 5-117). There were 42 (28%) cases with high grade and 110 (72%) with low grade tumor. Vascular invasion was found in 62 (41%) cases.

MMP-2 expression and correlation with other clinicopathological parameters

MMP-2 immunoreactivity presented as diffuse cytoplasmatic staining in tumor cells along the line of tumor invasion and in tumor cells within lymphatic/blood vessels. We found some cytoplasmatic MMP-2 positivity in endothelial cells, tumor associated macrophages, and fibroblasts of the stromal component. MMP-

Table 1. The association between overexpression of matrix metalloproteinase-2 (MMP-2) and clinicopathological variables in 152 patients with Duke's B colon cancer

	No. (%) of patients with MMP-2 expression†			
Variable	negative	positive	χ^2	*P*
Sex:			4.8	0.028
men	36 (38)	59 (62)		
women	32 (56)	25 (44)		
Age (years):			0.335	0.930
≤60	32 (49)	33 (51)		
>60	36 (41)	51 (89)		
Tumor differentiation:*			29.1	<0.001
high grade	4 (10)	38 (90)		
low grade	64 (58)	46 (42)		
Vascular invasion:			62.1	<0.001
yes	4(6)	58 (94)		
no	64 (71)	26 (29)		

*According to College of American Pathologists Consensus Statement 1999 (9).
†Staining intensity: negative –<10% cells with positive staining; positive –≥10% cells with positive staining (7,10).

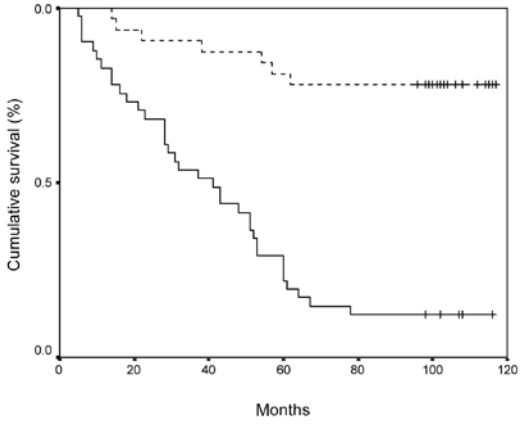

Figure 1. Overall survival and matrix metalloproteinase-2 (MMP-2) immunohistochemical expression in 152 patients with Duke's B colon cancer. Full line – positive: ≥10% cells with positive staining; broken line – negative: <10% cells with positive staining. Statistical analysis was performed with Kaplan-Meier test.

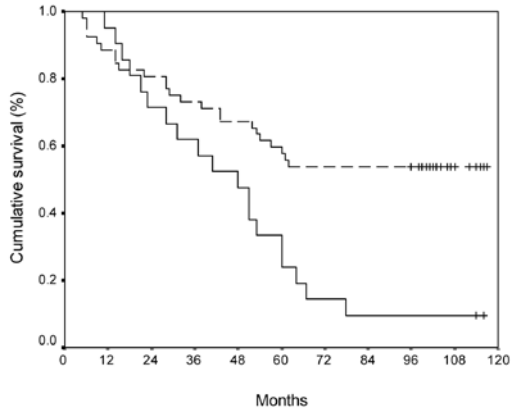

Figure 2. Overall survival and tumor differentiation in 152 patients with Duke's B colon cancer. Broken line – low grade tumor; full line – high grade tumor. Statistical analysis was performed with Kaplan-Meier test.

2 positive staining in ≥10% tumor cells was found in 84 (55%) cases.

A significant relationship was found between MMP-2 expression and other pathohistological parameters characteristic of aggressive behavior, such as high tumor grade and the presence of vascular invasion (Table 1). Univariate analysis confirmed that MMP-2 expression, high tumor grade, presence of vascular invasion, male sex, and age over 60 years were associated with the shorter overall survival (Table 2, Figures 1-4).

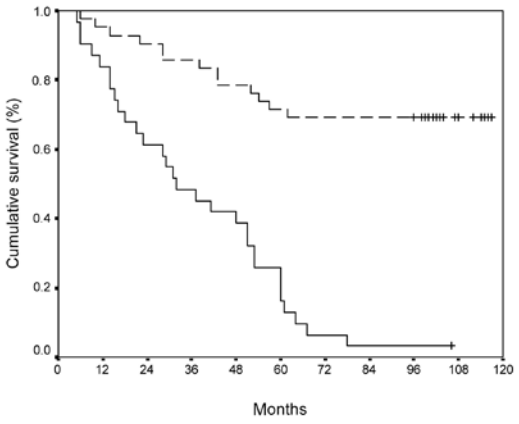

Figure 3. Overall survival and vascular invasion in 152 patients with Duke's B colon cancer. Broken line – tumor without invasion; full line – tumor with invasion. Statistical analysis was performed with Kaplan-Meier test.

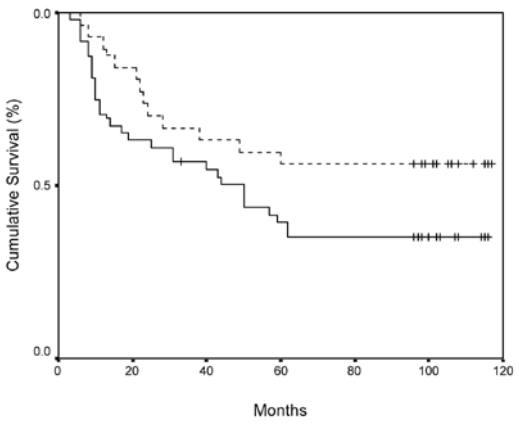

Figure 4. Overall survival and sex in 152 patients with Duke's B colon cancer. Broken line – women; full line – men. Statistical analysis was performed with Kaplan-Meier test.

Cox multivariate analysis for overall survival showed that only vascular invasion and positive MMP-2 expression were connected with shorter overall survival (Table 2).

Discussion

Our results showed that immunohistochemically detected high expression of MMP-2 is a predictor of poor prognosis in patients with Duke's B colon cancer.

The benefit of postoperative adjuvant chemotherapy in patients with Duke's B colon

Table 2. Univariate and multivariate analysis of effect of matrix metalloproteinase-2 (MMP-2) overexpression, patients' characteristics, and tumor parameters on overall survival in 152 patients with Duke's B colon cancer*

Parameter	Overall survival (SE)	Univariate analysis (Kaplan-Meier test)		Cox proportional hazard regression analysis		
		log rank	*P*	HR	95% CI	*P*
Sex:		5.5	0.019	0.63	0.37-1.08	0.094
women	77 (6)					
men	57 (5)					
Age:		6.8	0.009	1.16	0.68-1.97	0.59
≤60	85 (5)					
>60	63 (4)					
Tumor differentiation:†		20.1	<0.001	0.61	0.36-1.04	0.068
low	77 (4)					
high	48 (4)					
Vascular invasion:		72,0	<0.001	4.27	2.23-8.12	<0.001
no	92 (4)					
yes	38 (3)					
MMP-2:‡		64.8	<0.001	3.64	1.79-7.36	<0.001
negative	100 (4)					
positive	45 (4)					

*Abbreviations: SE – standard error; HR – hazard ratio; CI – confidence interval.
†According to College of American Pathologists Consensus Statement 1999 (9).
‡Staining intensity: negative –<10% cells with positive staining; positive –≥10% cells with positive staining (7,10).

cancer is still uncertain and its routine use is not recommended (11). Therefore, prognostic biomarkers may be useful for identifying high-risk patients with resected, node-negative disease. Furthermore, indicators of biologic behavior in colon cancer may help supplement the staging system and provide a basis for the implementation of novel therapeutic strategies targeting specific tumor-associated molecules according to individual tumor biology.

Most studies on MMP-2 protein expression investigated colon and rectal cancer together (2,12-16), although their biological features are heterogeneous (17,18), or only rectal cancer (10). Moreover, almost all studies included patients in all clinical stages (2,12-16).

To our knowledge, this is the first study that used immunohistochemical methods to determine the expression of MMP-2 in the subpopulation of patients with Duke's B colon cancer and investigated its relationship with clinicopathological parameters and patient survival.

Previous studies on MMP-2 protein expression as a prognostic marker in colon carcinoma used various methods, including gelatin zymography (19), enzyme linked immunosorbent assays (19), quenched fluorescence substrate hydrolysis (20), Western blot (19), in situ hybridization (19), and immunohistochemistry on formalin fixed, paraffin embedded tissue (17,18). Immunohistochemistry has several advantages over other methods – it allows direct correlation with morphology and it can be performed on paraffin embedded specimens, which makes it practical for the routine assessment of MMPs in diagnostic practice. However, it has been suggested that it cannot distinguish between latent and activated forms of MMPs (21). On the other hand, other techniques, such as zymography or quenched fluorescent substrate hydrolysis, are able to distinguish between the latent and active forms of MMP-s, but they do not allow a correlation with morphology (6,20).

We found the positive expression of MMP-2 protein in 55% of samples, which is in line with other studies, where MMP-2 expression varied from 35% (10) to 68.8% (22).

MMP-2 immunoreactivity presented as a diffuse cytoplasmatic staining in tumor cells along the line of tumor invasion and in tumor cells within lymphatic/blood vessels, similar to other studies where increased levels of MMP-2 protein were observed in the invasive region of colorectal tumors (23). This is in line with the hypothesis that MMP-2 provides a phenotypic hallmark of invasive potential and supports

the fact that the balance between proteases and antiproteases at the invading edge of the tumor is disrupted in favor of proteases (4).

We found some cytoplasmatic MMP-2 positivity in endothelial cells, tumor associated macrophages, and fibroblasts of the stromal component, which confirms the earlier reports by Kikuchi (14) and Schwander (10).

Tumor cells interact with stromal cells in the metastatic cascade. After attachment to the components of the basement membrane, tumor cells secrete MMPs and other proteolytic enzymes or induce host cells to produce proteases (4).

Positive immunostaining of MMP-2 significantly correlated with the presence of vascular invasion. These results are in concordance with other studies (14,15,24) and support the claim that MMP-2 has the key role in local expansion and infiltration of the tumor cell mass, as well as metastatic invasion.

MMP-2, together with other MMPs, degrades collagen type IV and mobilizes vascular endothelial growth factor that is sequestered in the basement membrane. Degradation of collagen IV also exposes normally cryptic domains of the protein, which serves as an important signal for angiogenesis. Finally, tumor gains access to the circulation by penetrating the vascular basement membrane (4).

Similar to studies by Ring (16) and Papandopoulou (25), we found higher MMP-2 expression in high-grade tumors, although some studies show the opposite finding (13,19). Since tumor grade and vascular invasion are well-known prognostic factors (26), their correlation with MMP-2 positive immunostaining puts them in the group of pathohistological parameters characteristic for aggressive tumor behavior.

Univariate analysis showed that MMP-2 expression, high tumor grade, the presence of vascular invasion, male sex, and age over 60 years were associated with shorter overall survival in patients with Duke's B colon cancer. Other studies also showed that high tumor grade and presence of vascular invasion were stage-independent prognostic factors in colorectal carcinomas (9,26,27).

Sex-related survival differences in patients with colorectal cancer were found in only two studies (28,29). Press et al (28) explained these differences by the fact the colon expresses both estrogen receptor β and androgen receptor, and that epidermal growth factor receptor (EGFR), the high expression of which is linked with poor prognosis, interacts with both. They suggested that EGFR may have molecular intermediates that interact in a sex-specific way to affect EGFR pathway activation.

We found that both vascular invasion and MMP-2 were independent significant factors related to the overall survival. However, there are some limitations to our study, such as the relatively small number of respondents and the use of only immunohistochemical method for the detection of MMP-2 protein expression. Therefore, further studies on the impact of MMP-2 on the prognosis are needed. Such studies should include a homogenous and sufficiently large patient-population, have a sufficiently long follow-up period, and investigate colon and rectal cancer separately.

According to our results, the independent prognostic factor MMP-2 should be taken into consideration in decision making on the use of adjuvant systemic therapy in patients with Duke's B colon cancer.

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