Pathol. Oncol. Res. (2008) 14:31–37
DOI 10.1007/s12253-008-9004-5

ORIGINAL PAPER

Matrix Metalloproteinase-9 Expression in the Normal Mucosa–Adenoma–Dysplasia–Adenocarcinoma Sequence of the Colon

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Received: 28 January 2008 / Accepted: 5 February 2008 / Published online: 18 March 2008

Abstract It has been proposed that matrix metalloproteinases (MMPs) play a role in tumor invasion. We determined protein expression of matrix metalloproteinase-9 (MMP-9) in colorectal cancer (CRC), corresponding normal mucosa and colorectal adenomas. For confirmation of immunohistochemical results MMP-9 TaqMan RT-PCR analysis was performed. Expression of MMP-9 was determined on paraffin embedded biopsy sections by immunohistochemistry in 31 CRC patients (from cancer tissue and corresponding normal mucosa) and in 30 patients with adenoma (nine adenomas with high grade of dysplasia). MMP-9 immunostaining was determined semi-quantitatively. For Taqman RT-PCR analyses normal mucosa (n=5), adenoma without (n=6) and with high grade dysplasia (n=7) and CRC (n=10) were investigated. Statistical analysis with ANOVA, LSD test and correlation analysis were performed. P value of <0.05 was considered significant. The MMP-9 expression in CRC was significantly higher compared to adenomas or the normal mucosa (P= 0.001). Significantly higher expression of MMP-9 has been observed in adenomas with high grade dysplasia compared to other adenomas or normal colon (P<0.001). Diffuse strong MMP-9 expression was present in tumor as well as in stromal cells. In adenoma samples, dysplastic epithelial cells showed moderate intensive cytoplasmic MMP-9 expression, with a clear-cut differentiation between dysplastic and non-dysplastic areas. Staining intensity correlated with the grade of CRC. We demonstrate a significantly higher expression of MMP-9 in adenoma with high grade dysplasia—CRC sequence as compared to normal tissue. The over-expression of MMP-9 strongly suggests its association with colorectal carcinogenesis.

Keywords Adenoma · Colorectal cancer · Dysplasia · Matrix metalloproteinase-9 · Polymerase chain reaction

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Introduction

Colorectal cancer (CRC) is the most common gastrointestinal cancer in the Western world and it is the second leading cause of cancer-related mortality in developed countries [1–3].

Proteolytic enzymes are thought to be major contributors to the breakdown and reconstitution of extracellular matrix (ECM) in physiological processes, like tissue remodeling during development, growth and wound repair, and in pathological conditions, including destructive diseases and tumor progression. Tumor cells have been shown to produce and release several proteolytic enzymes, which are thought to be involved in tumor invasion and metastasis [4, 5]. Several human solid tumors have been reported to have increased levels of proteolytic enzymes in cancer tissue, strongly suggesting that proteases may be important in tumor invasion and metastasis. We have previously

demonstrated that proteolytic enzymes, such as cysteine and serine proteases, are widely distributed in gastrointestinal tissues, being implicated in processes of gastrointestinal tissue remodeling and angiogenesis [6]. Furthermore, may have a role not only in the process of gastric [7, 8] or colorectal cancer invasion [9], but also in the progression of gastrointestinal precancerous changes into cancer [10].

Matrix metalloproteinases (MMPs) are genetically distinct but structurally related matrix-degrading enzymes. MMPs can collectively degrade almost all extracellular matrices and basement membrane proteins [11–13]. MMPs are classified as gelatinases, collagenases, stromelysins, membrane-type matrix metalloproteinases, based mainly on the in vivo substrate specificity of the individual MMP [14, 15]. Type IV collagen is an important protein of the basement membrane. We and others have reported that type IV collagenase, matrix metalloproteinase-9 (MMP-9; gelatinase B) was expressed in several gastrointestinal tumors and especially important in the process of tumor invasion and metastasis [16–18]. CRC is characterized by enhanced expression of MMP-9 [19–25], but also of several other MMPs, such as MMP-1, MMP-2, MMP-7 or MMP-13 [26–29]. Some studies have also pointed to the possible prognostic value of MMP-9 in CRC [30–35].

To our knowledge however, MMP-9 expression has not been evaluated in the development and progression of normal mucosa–adenoma–dysplasia–adenocarcinoma sequence in the colon. Hence, the aim of the present study was to investigate the behavior of MMP-9 expression in relation to whole sequence of adenoma–dysplasia–CRC sequence using immunohistochemical and TaqMan real-time polymerase chain reaction (RT-PCR) analyses and to evaluate any correlation between the MMP-9 expressions and clinicopathological staging of CRC.

Materials and Methods

Tissue specimens were obtained endoscopically from in- and outpatients with abdominal complaints at the 2nd Department of Medicine, Semmelweis University Budapest. After informed consent, during the routine colonoscopies, colorectal biopsy samples were taken from 31 CRC patients (from cancer tissue and corresponding normal mucosa; 11 males and 20 females, mean age 61±6.7years; range, 47–74years). For further comparison we investigated 30 patients with colorectal adenoma confirmed by histology after endoscopic polypectomy. Samples consisted of 11 tubular adenomas and 10 tubulovillous adenomas with low grade dysplasia and nine tubulovillous adenomas with high grade dysplasia (13 males and 17 females, mean age 58.2± 6.5years, range 43–68years).

In all instances, this was the first diagnosis of CRC, and no recurrences were taken into consideration. Clinical data for the patients and histologic data for the tumors were registered accurately. Pathologic staging was obtained for the presence (n = 17) or absence (n = 14) of lymph node and/or distant metastases; and for differentiation [well differentiated, G1 (n = 5); moderately differentiated, G2 (n = 17), or poorly differentiated, G3 (n = 9)].

The tumors were histologically classified according to Dukes classification [36], as modified by Turnbull *et al.* [37]. Dukes stage A tumors are confined to the bowel wall (n = 6); Dukes stage B tumors have spread beyond the wall without involving lymph nodes (n = 8); Dukes stage C are associated with regional lymph node metastases (n = 7); and finally, Dukes stage D tumors are associated with distant metastases (n = 10).

For Taqman RT-PCR analyses, using an independent set of samples, normal healthy mucosa (n = 5), colorectal adenomas (n = 13; six colorectal adenomas without dysplasia and seven adenomas with high grade dysplasia) and CRC (n = 10) were investigated (the CRC cases were classified as Dukes stage B, n = 6, and Dukes stage D, n = 4).

MMP-9 Immunohistochemistry

The endoscopic specimens were fixed in formalin and embedded in paraffin wax, sliced in serial step sections of 4μm thickness. To assess the location of MMP-9 within the intestinal tissues, immunohistochemical staining for the MMP-9 was performed as described previously [18]. Briefly, after dewaxing in xylene and rehydration through graded ethanol, endogenous peroxidase activity was blocked by incubation for 30min at room temperature in 3% hydrogen peroxide. After PBS washing, non-specific blocking was done with 1% BSA–PBS solution for 10min at room temperature. Then the slides were incubated with optimally diluted monoclonal anti-human MMP-9 antibody (Clone, 36020.111, R&D Systems) at 37°C for 60min in a humidified chamber. After washing them three times in PBS, signal conversion was carried out with the LSAB2 system (DAKO) as described in the manual. Haematoxylin co-staining was done. Tissue sections from human ovarial carcinoma were used as positive and negative controls.

Immunohistochemical Analysis of MMP-9

Known immunohistochemically positive tissue sections were used as positive controls, and negative control sections were processed immunohistochemically after having replaced the primary antibody by PBS. None of these negative control sections exhibited immunoreactivity. Immunostaining was determined semiquantitatively, as described previously [18]. Essentially, the intensity of staining for MMP-9 under a light

microscope was graded from 0 to 3, denoting no staining or light, moderate, or intense staining. An immunohistochemical staining score was calculated for each histologic area by multiplying the staining intensity level (0 to 3) by the proportion of cells in each area staining with that intensity. The immunohistochemical staining score for an area with 100% of cells staining with 3 intensity, for example, would be 1 × 3, equaling 3, whereas an area with 50% cells staining 2 and 40% staining 1 would have a score of 0.5 × 2 plus 0.4 × 1, equaling 1.4. Two independent investigators without knowledge of the clinical outcomes evaluated the degree of immunohistochemical staining intensity. There was less than 5% variance between the results of two counts.

Taqman RT-PCR

RT-PCR was performed as described previously [38]. MMP-9 (TaqMan probe ID, Hs00957562_m1) TaqMan real-time PCR was used to measure the mRNA expression of the observed parameter using an Applied Biosystems Micro Fluidic Card System. The measurements were performed using an ABI PRISM® 7900HT Sequence Detection System as described in the products User Guide (http://www.appliedbiosystems.com, CA, USA). The total cycle number was 45.

Statistical Analysis

Statistical analysis with one-way ANOVA (analysis of variance), LSD (least significant difference) test were performed by the Statistica for Windows 4.3 program package. Due to the high standard deviations of some of the series, the immunohistochemical expression scores of MMP-9 were expressed as mean ± standard error of mean (SEM). P value of <0.05 was considered significant.

In case of Taqman RT-PCR, for data analysis the SDS 2.2 software was used. The extracted delta CT (dCT) values (which represent the expression normalized to the ribosomal 18S expression) were grouped according to the histologic groups. Then the Student's t test was performed to compare the expression values between groups.

Results

The immunohistochemical expression scores of MMP-9, expressed as mean ± SEM in normal colonic mucosa (n = 31), colorectal adenomas (n = 30) and CRC (n = 31) are shown in Table 1.

The semiquantitative score of MMP-9 in CRC was significantly higher compared to colorectal adenomas or the normal mucosa (P<0.001). Colorectal adenomas expressed higher MMP-9 expressions than the normal colonic mucosa, but the difference was not statistically significant. Significantly higher expression levels of MMP-9 have been observed in cases of tubulovillous adenomas with high grade dysplasia (n = 9) compared to the other adenomas (tubular and tubulovillous adenomas with low grade dysplasia; n = 21; 1.83±0.27 vs 0.38±0.08, P<0.001) or to the normal colonic mucosa (1.83±0.27 vs 0.46±0.12, P<0.001).

Table 1 Immunohistochemical expression of matrix metalloproteinase-9 (MMP-9) in normal colonic mucosa, colorectal adenoma and colorectal carcinoma according to a semiquantitative score (mean ± standard error of mean, SEM)

Histology	Score (mean ± SEM)
Normal colon (n = 31)	0.46±0.12
Colorectal adenoma (n = 30)	0.81±0.15
Colorectal carcinoma (n = 31)	1.98±0.13
ANOVA one-way	P<0.0001

In normal colonic epithelium minority of the crypt epithelial cells showed MMP-9 cytoplasmic expression, mainly near to the luminal surface. In the lamina propria some leukocytes and myofibroblasts were found to show diffuse cytoplasmic MMP-9 staining (Fig. 1a). In adenoma samples, the majority of dysplastic epithelial cells showed moderate intensive cytoplasmic MMP-9 expression, with a clear-cut differentiation between dysplastic and non-dysplastic areas (Fig. 1b). Further, CRC tumor cells as well as stromal cells expressed MMP-9 staining, denoting a diffuse strong MMP-9 expression in tumor cells. MMP-9 was mainly expressed within the cytoplasm and cytoplasmic membranes of the colonic epithelium in adenocarcinoma cells. The surrounding lamina propria was filled with MMP-9 positive leukocytes and myofibroblast like cells (Fig. 1c).

With respect to tumor differentiation (grading), expression of MMP-9 was significantly higher in moderately (G2) or poorly (G3) differentiated tumors than in well differentiated (G1) cancers (P = 0.02; Table 2).

No statistically significant differences were observed with respect to the immunoexpression of MMP-9 in correlation with the presence or absence of metastases (data not shown).

With respect to Dukes classification, MMP-9 immunoexpression was slightly higher in Dukes stages B, C and D than in Dukes stage A, but the differences were not statistically significant (Table 3).

Taqman RT-PCR

For confirmation of immunohistochemical results MMP-9 TaqMan real-time RT-PCR analysis was performed. The EV in CRC were significantly higher compared to the EV of normal colonic mucosa (P<0.05; Table 4). Colorectal

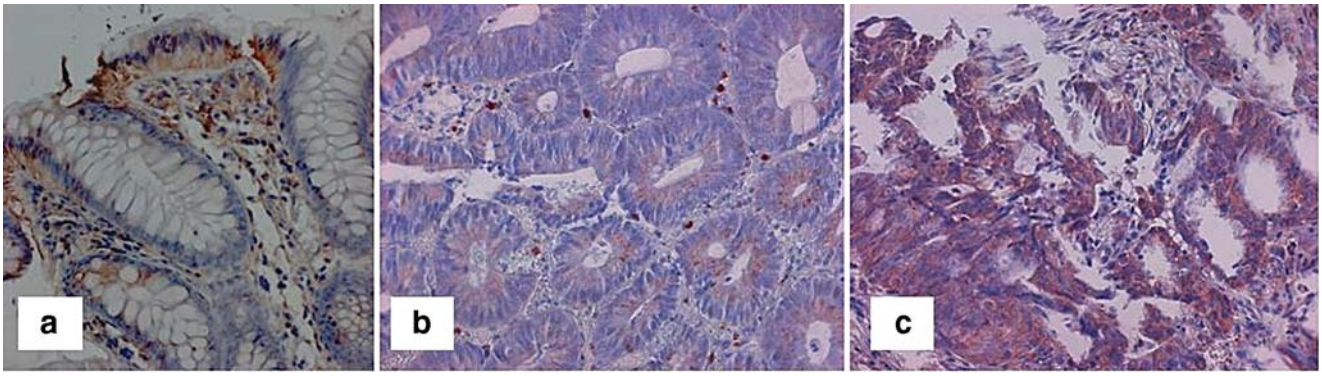

Fig. 1 Expression of MMP-9 in different colonic tissues. **a** Normal healthy mucosa, **b** colorectal adenoma with low-grade dysplasia, **c** colorectal carcinoma (MMP-9-biotin-streptavidin-amino-aethyl-carbasole; hematoxylin; ×200 magnification). **a** In normal colonic epithelium minority of the crypt epithelial cells shows MMP-9 cytoplasmic expression, mainly near to the luminal surface. In the lamina propria some myofibroblasts and leukocytes were found to show diffuse cytoplasmic MMP-9 immunoreactivity. **b** In adenoma samples, the majority of the dysplastic cells show moderate intensive cytoplasmic MMP-9 expression. In the lamina propria, the number of MMP-9 positive cells was a bit higher than in normal colonic samples. **c** In colorectal carcinoma samples diffuse strong cytoplasmic MMP-9 expression is present in epithelial tumor cells. The surrounding lamina propria was filled with MMP-9 positive leukocytes and myofibroblast like cells

adenomas showed a trend toward higher MMP-9 expression than the normal colonic mucosa, but the difference was not statistically significant.

According to Taqman RT-PCR analyses both Dukes stage B (EV, 27.634±1.231) and Dukes stage D tumors (EV, 27.289±0.534) showed significantly higher MMP-9 expression than the normal colonic mucosa (EV, 26.112± 0.88; $P<0.05$).

Discussion

Tumor cell invasion and metastasis are regarded as multi-step phenomena, involving the proteolytic degradation of the basement membrane and the ECM. Among the many steps in invasion and metastasis, excessive degradation of the ECM is one of the hallmarks of this process [4, 5]. Many proteolytic enzymes are capable of degrading ECM components. We have previously reported that proteolytic enzymes, such as cysteine and serine proteases are involved not only in processes of gastrointestinal tissue remodelling and angiogenesis, but also in the process of gastric or colorectal carcinogenesis and invasion [6–10]. However, the proteinase system primarily responsible for ECM degradation in vivo is MMPs. MMPs have been closely linked to the invasiveness and metastatic phenotype of cancer cells [11–15]. MMP-9 is expressed in several gastrointestinal tumors [16–18] and promotes cancer progression by regulating physiologically based processes co-opted during metastasis, including cell adhesion, migration, tissue invasion, intravasation, extravasation and angiogenesis [39–41]. MMP-9 has also been implicated to play a role in CRC progression, invasion and metastasis in animal models as well as in patients [42].

Given the lack in the literature of the evaluation of MMP-9 expressions in normal mucosa –adenoma–dysplasia–adenocarcinoma sequence in the colon, we investigated the behavior of MMP-9 expression in this sequence using immunohistochemical and TaqMan RT-PCR analyses. In the present study, mucosal expression of MMP-9 was significantly higher in CRC than in corresponding paired normal mucosa, thus confirming previous observations that CRC is characterized by enhanced expression of MMP-9 [19–25]. In addition, we can confirm the previous immunohistochemical results showing that tumor epithelial and stromal cells express equivalent quantities of MMP-9 [19,

Table 2 Immunohistochemical expression of matrix metalloproteinase-9 (MMP-9) with respect to the differentiation (grade) of colorectal carcinoma according to a semiquantitative score (mean ± standard error of mean, SEM)

Differentiation	Score (mean ± SEM)
Well differentiated, G1 (n = 5)	1.30±0.20
Moderately differentiated, G2 (n = 17)	2.11±0.14
Poorly differentiated, G3 (n = 9)	2.33±0.28
Kruskall–Wallis analysis of variance	P = 0.02

Table 3 Immunohistochemical expression of matrix metalloproteinase-9 (MMP-9) in association with Dukes classification of colorectal cancer according to a semiquantitative score (mean ± standard error of mean, SEM)

Stage	Score (mean ± SEM)
Dukes A (n = 6)	1.58±0.32
Dukes B (n = 8)	2.25±0.25
Dukes C (n = 7)	1.85±0.26
Dukes D (n = 10)	2.00±0.21
ANOVA one-way	P = not significant

Table 4 Taqman RT-PCR analyses of matrix metalloproteinase-9 (MMP-9) expression in normal colonic mucosa, colorectal adenoma and colorectal carcinoma, expressed as dCT and Expression Values (EV; mean ± standard deviation)

Histology	dCT	EV (45-dCT)
Normal colon (n = 5)	18.888±0.880	26.112±0.88
Colorectal adenoma (n = 13)	18.658±0.985	26.342±0.985
Colorectal carcinoma (n = 10)*	17.538±0.882	27.462±0.882

dCT extracted delta CT, *EV* expression values (total cycle number−dCT)
*P<0.05 vs normal colon (statistics)

20, 42–44]. Our results demonstrate that in adenomas the majority of dysplastic epithelial cells show moderate intensive cytoplasmic MMP-9 expression, with a clear-cut differentiation between dysplastic and non-dysplastic areas. As shown by others, in normal colonic mucosa the prominent location of MMP-9 was in the submucosa and a pronounced positive staining for MMP-9 in leukocytes and myofibroblasts is observed [45, 46].

We have previously demonstrated that proteolytic enzymes, such as cysteine and serine proteases are implicated in the progression of gastrointestinal precancerous lesions into cancer [10]. We and others have reported that MMPs are overexpressed in a variety of premalignant tumor tissues, including Barrett's esophagus, colorectal adenoma or inflammatory bowel disease [18, 47, 48]. Our immunohistochemical analysis revealed a progressive increase in expression of MMP-9 with increasing severity of lesions of the colon. MMP-9 expression was significantly lower in normal colonic epithelium compared to the other groups. Significantly higher expression levels of MMP-9 have been observed in colorectal adenomas and concomitant high grade dysplasia compared to normal healthy mucosa. Finally, we observed that MMP-9 expression was significantly higher in CRC compared to adenomas. These results suggest that overexpression of MMP-9 plays an important role in the progress in to CRC, and MMP-9 protein may serve as a marker for invasiveness. Our results indicate that activation of MMP-9 may be an early event in colorectal carcinogenesis.

With respect to the correlation between MMP-9 expression and clinicopathological staging of CRC, our data are in agreement with some previously published results, suggesting the possible prognostic value of MMP-9 in CRC [30–35]. The finding in our study of significantly higher MMP-9 expression in tissue samples from patients with poorly differentiated (G3) or moderately (G2) differentiated tumors compared to well differentiated (G1) cancers confirmed that MMP-9 is involved in CRC progression. In our series the differences were not statistically significant with respect to Dukes classification, however MMP-9 expression was slightly higher in advanced Dukes stages than in Dukes stage A.

For confirmation and validation of immunohistochemical results MMP-9 TaqMan RT-PCR analysis was performed in an independent set of samples. Our RT-PCR results correlate with the immunohistochemical behavior of MMP-9 in the colonic mucosa, showing a significantly higher expression of MMP-9 in cancer tissue compared to normal colonic mucosa.

However, there is a discrepancy in the MMP-9 protein and mRNA levels of adenomas versus normal mucosa. This could be explained partly by the relative small number of patients examined in each group. On the other hand, the differences between the MMP-9 protein and mRNA levels could be explained by the clear-cut differences in the methodology. During immunohistochemistry we used localisation specific, semiquantitative parametric method, whereas during RT-PCR technology the results were dependent on the overall weight of the biopsy specimens and on the number of adenomatous cells examined. These methodological differences probably could explain why colorectal adenomas showed only a trend toward higher MMP-9 expression than the normal colonic mucosa, but the difference was not statistically significant using Taqman RT-PCR; in contrast, the semiquantitative immmunohistochemical expression of MMP-9 was significantly higher in cases of colorectal adenomas compared to the normal colonic mucosa.

On the other hand, the differences concerning MMP-9 protein and mRNA behaviour in adenoma/CRC sequence could also have biological explanations. One of the possible biological mechanisms could be that at early stages of carcinogenesis several epigenetic mechanisms (i.e. DNA methylation status; alterations of K-ras proto-oncogene signalling pathway; methylation status of APC) are involved [49–51]. Furthermore, the role of naturally occurring tissue inhibitors of MMP-s (TIMPs) might also be taken into consideration. Several investigators have demonstrated that TIMPs have growth-promoting properties, and recent experimental findings support the hypothesis that TIMPs might also stimulate tumor growth by inhibiting apoptosis [52–54].

Our findings are relevant from both, biological and clinical point of view. Despite the advance in preoperative and postoperative medical care of CRC patients, their prognosis has not improved significantly. Therefore, it would be useful to have additional biomarkers to help clinicians better determine the risk of CRC development. It has been suggested that determination of MMP-9 expression could be useful for monitoring the efficacy of chemotherapy. Indeed, it has been demonstrated that the efficacy of postoperative adjuvant chemotherapy is less effective for patients with a CRC positive for MMP-9 having a greater risk for postoperative recurrence [55]. Furthermore, the plasma levels of MMP inhibitor (TIMP-1)

are significantly associated with objective response and survival in metastatic CRC patients receiving combination chemotherapy [56]. One can speculate that the relevance of MMP-9 in colorectal carcinogenesis also may support a possible therapeutic approach. MMP-9 can offer an ideal candidate for targeted antimetastatic therapy in CRC [44, 57–59]. Indeed, this can be obtained directly by inhibition of MMP-9. Phase I-III clinical trials with matrix metalloproteinase inhibitors are under evaluation [60–62].

In conclusion, our results demonstrate a significantly higher expression of MMP-9 in adenoma–high grade dysplasia–adenocarcinoma sequence as compared to normal colonic tissue. The overexpression of MMP-9 strongly suggests its association with colorectal carcinogenesis. Gain of MMP-9 in adenomas with high grade dysplasia compared to other adenomas indicates that this alteration may be an early event in colorectal carcinogenesis. Together with other biological markers quantification of MMP-9 might be useful to identify patients at higher risk for progression to CRC.

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