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Up-regulation of gelatinases in the colorectal adenoma-carcinoma sequence

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ABSTRACT

Gelatinase activity has been associated with colorectal cancer (CRC) invasion and metastasis. However, it remains unresolved whether these proteases participate in early colorectal carcinogenesis. The activity of metalloproteinases (MMP) 2 and 9 were measured by zymography in 122 colorectal adenomas, 22 CRC samples, 12 hyperplasic polyps and in 114 matched normal mucosal samples from 114 patients undergoing colonoscopy. There was a progressive and significant increase of pro-MMP-9 activity from adenoma to CRC tissue, whereas the activity of the latent and active forms of MMP-2 was exclusively up-regulated in CRC samples. Among neoplastic polyps, pro-MMP-9 activity was significantly higher in advanced versus non-advanced adenomas and in those harbouring high grade dysplasia. In addition, a positive correlation was observed between MMP-9 activity and the size of the adenomas. The present study demonstrates that MMP-9 is markedly up-regulated in the adenomatous tissue and suggests that this gelatinase might be a marker for early colorectal carcinogenesis.

1. Introduction

Matrix metalloproteinases (MMP) are a group of zinc-dependent matrix degrading enzymes that play an important role in the pathogenesis of various inflammatory diseases including rheumatoid arthritis, osteoarthritis, atherosclerosis, cancer and inflammatory bowel disease.[1] MMP activity is the result of the balance between the concentration of their active forms and the presence of tissue inhibitors of MMP (TIMPs). Although MMP have been divided into five groups (gelatinases, collagenases, stromelysins, matrilysins and membrane MMP) depending on the substrate specificity, the MMP system has shown to be very redundant, and each MMP is able to break down more than one extracellular matrix component (ECM).[2]

Degradation of ECM components by MMP contributes to the removal of the physical barriers to cancer, a fact that occurs at several stages of disease progression including local invasion, neoangiogenesis and extravasation. Therefore, several MMP have been involved in tumour progression, either in the invasive behavior or the ability to metastasise.[3] Epidemiological studies have examined the expression and prognostic value of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in patients with colorectal cancer (CRC).[4–17] Both gelatinases

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are predominantly synthesised in the stroma and their main function is to degrade type IV collagen.[4,5] Their expression has been related to basement membrane loss[6] which has been associated with an increased frequency of distant metastases.[6–9] An enhanced MMP-9 expression in the tumour compared to normal tissue has been correlated with increased disease recurrence and reduction of overall survival.[17] Therefore, there is abundant evidence demonstrating the pathogenic role of these MMP at the late stage of colorectal carcinogenesis. By contrast, the expression of these gelatinases in early colorectal neoplasms (advanced adenoma or early carcinoma) is limited and data are inconsistent.[10–16] Some studies have shown over-expression of these enzymes in adenomatous polyps,[10,13] whereas others observed very weak or no expression at all.[12,15] In fact, no data exists on the expression of gelatinases in advanced versus non-advanced adenomas or in lesions with potential risk of malignisation such as flat-elevated or flat-depressed lesions. Therefore, the aim of the present study was to assess whether gelatinase activity is up-regulated in the early stages of the colorectal adenoma-carcinoma sequence.

2. Materials and methods

2.1. Patients

In this prospective study a total of 114 patients were included. Ninety two patients scheduled to undergo a clinically indicated colonoscopy and diagnosed with colorectal polyps and 22 patients diagnosed with CRC. Colorectal polyps were classified according with their size (equal or larger than 1 cm or smaller than 1 cm) and shape (flat or protruded). The Japanese Research Society classification was employed to classify colorectal lesions in flat (elevated or depressed) and protruding.[18] Sawada's criteria were used to define flat polyp (height less than half the diameter of the lesion).[19] In addition, neoplastic colorectal polyps were classified as advanced ($\geqslant$1 cm, presence of high grade dysplasia or villous or tubulo-villous glandular pattern) or non-advanced adenomas, as the former are a valid surrogate marker of CRC risk.[20] Indications for colonoscopy and the baseline characteristics of the subjects are listed in Table 1 Patients with inflammatory bowel disease (where MMP activity may be increased) or hereditary polyposis syndromes were not included in the study.

Table 1 – Demographic data and indication for colonoscopy

Characteristics	*n*	(%)
Men	76	67
Women	38	33
Age (mean ± SD), yr	64 ± 11	
Indication for colonoscopy		
History of polyps	28	25
Hematochezia	20	18
Anaemia	25	22
Screening	16	14
History of colon cancer	7	6
Change in bowel habits	5	4
Abnormal barium enema	6	5
Abdominal pain	4	4
Family history colonic neoplasm	3	3

2.2. Colonoscopic procedures and tissue collection

Colonoscopic examinations were performed with conventional (CF-140 L) colonoscopes (Olympus Corp., Tokyo). Whenever a flat lesion was suspected 0.5% indigo carmine was directly applied with a syringe. Polyp size was determined by comparison of the lesion with fully opened biopsy forceps. Lesions were sampled either with a biopsy forcep before polypectomy, or with a scalpel after polypectomy. Flat lesions were usually resected by mucosectomy; samples from such lesions were taken with the aid of a dissecting microscope.

Paired samples of all lesions and normal surrounding tissue were collected. One of each pair was used for pathological diagnosis on hematoxylin/eosin stained paraffin sections and the other was frozen in liquid nitrogen and stored at −80 °C until used for protein extraction as described later. The following data were collected: shape, size, histology (neoplastic or hyperplastic), level of dysplasia, histologic phenotype. Biopsy specimens were collected with consent from patients. The protocol study was approved by the Ethics and Clinical Research Committee of University Hospital of Canary islands.

2.3. Tissue preparation

Biopsy specimens (3–5 mg) were homogenised in extraction buffer (50 mmol/L TrisHCl pH = 7.6, 150 mmol/L NaCl) and protease inhibitors (Complete, Roche), sonicated two times for 10 s (UP100H, Dr Hielscher, Germany) at 1 min intervals and, after 10 min on ice, protein extracts were centrifuged at 10,000 g for 10 min. The supernatants were removed and assayed for protein concentration (Bicinconinic acid, Sigma, St Louis). Homogenates were stored at −80 °C until assayed.

2.4. Gelatinase activity

The activity of both gelatinases was analysed by zymography on tissue homogenates, as previously described.[14] Both active and pro-form MMP-2 and the pro-form of MMP-9 were determined, as the active form of MMP-9 (82 kDa) is rarely detectable by zymography.[5] Briefly, proteins (25 μg per lane) were separated in 10% polyacrilamide gel electrophoresis (SDS-PAGE) containing 0.2% gelatin (Sigma, Co.) under non-reducing, non-denaturing conditions. Gelatinases were detected using a developing buffer (50 mmol/L TrisHCl pH = 7.5, 200 mmol/L NaCl, 10 mmol/L $CaCl_2$ and 1 μmol/L $ZnCl_2$). Serine protease inhibitors (2 μ g/mL aprotinin or 20 μ g/mL leupeptin) and MMP inhibitors (10 mmol/L EDTA or 0.1 mmol/L o-phenantroline) were added to the developing buffer, to assess whether the observed gelatinolytic bands were caused by serine protease activity or MMP activity.

Human MMP-9 and MMP-2 standards (Oncogene Research) were run as controls. After 18 h development, gels were fixed and stained in 40% methanol, 10% acetic acid and 0.1% (w/v) Coomassie Blue R-250 (Sigma) for 1 h and then distained.

Relative molecular weights of clear bands were analysed in comparison to molecular weight standards (DualColor, Bio-

Rad) and purified human MMP-2 and MMP-9 using a calibrated densytometer (GS-800, BioRad) and QuantityOne Quantitation analysis software (BioRad, version 4). Zymographic activity of each band was measured in terms of optical density/mm^2(OD/mm^2).

2.5. *Metalloproteinase-9 expression*

Since MMP-9 activity was markedly enhanced in homogenates of adenomatous tissue we tested the expression of this gelatinase by western blot analysis.[21] Samples were denatured in reducing treatment buffer and loaded (50 μg/lane) onto 10% polyacrylamide gels. After SDS-PAGE electrophoresis, proteins were transferred onto nitrocellulose membrane (Protran, Schleider& Schuell) and detected using a rabbit anti-MMP9 (Chemicon) at 1:1000 dilution, a matching secondary antibody (Jackson Immunoresearch) and a chemiluminiscent substrate (Pierce) according to the manufacturers instructions. The antibody binding was imaged on X-OMAT film (Kodak). Western blotting with monoclonal anti-alpha-tubulin (Sigma, Chemical, St Louis) was performed as internal control.

2.6. *Statistical analysis*

Results are expressed with the mean ± SD. The Kruskall–Wallis global test was used to compare the means of gelatinase activity depending on the type of tissue (normal, hyperplastic, non-advanced adenoma, advanced adenoma, or cancer). *A posteriori* comparisons were performed using the U-Mann Whithey test. *T*-test for independent samples was used to compare gelatinase activity depending on adenoma morphology (protruding versus flat or advanced versus non-advanced). The same test was used to compare advanced flat and advanced protruding adenomas. A p value lower than 0.05 (exact or not) was considered statistically significant. All data analyses were performed using SPSS v.12.0.1 (Chicago, IL) and StatXact 5.0.3 (Cambridge, MA).

3. Results

A total of 122 sporadic adenomas, 22 advanced CRC, 12 hyperplasic polyps and 114 paired normal colonic mucosal samples were analysed. On the whole, 63 (52%) adenomas were advanced (mean size, 17 ± 9 mm) and 59 (48%) non-advanced (mean size, 4 ± 2 mm). According to their shape, 69 (57%) adenomas were protruding (mean size, 11 ± 9 mm) and 53 (43%) flat (mean size, 10 ± 9 mm), including four flat-depressed lesions. Twenty-five flat (47%) and 38 protruding adenomas (55%) were advanced. Eleven lesions (9%) presented HGD in the histopathological analysis (four flat and seven protruding polyps). Eighty-nine (74%) adenomas were tubular, 27 (23%) tubulo-villous and four (3%) villous.

Fig. 1 demonstrates that bands observed by zymography correspond to MMP activity. Fig. 2 shows the level of MMP-2 activity at different tissues. The mean level of pro-MMP2 activity was significantly higher in CRC samples (61 ± 20 OD/

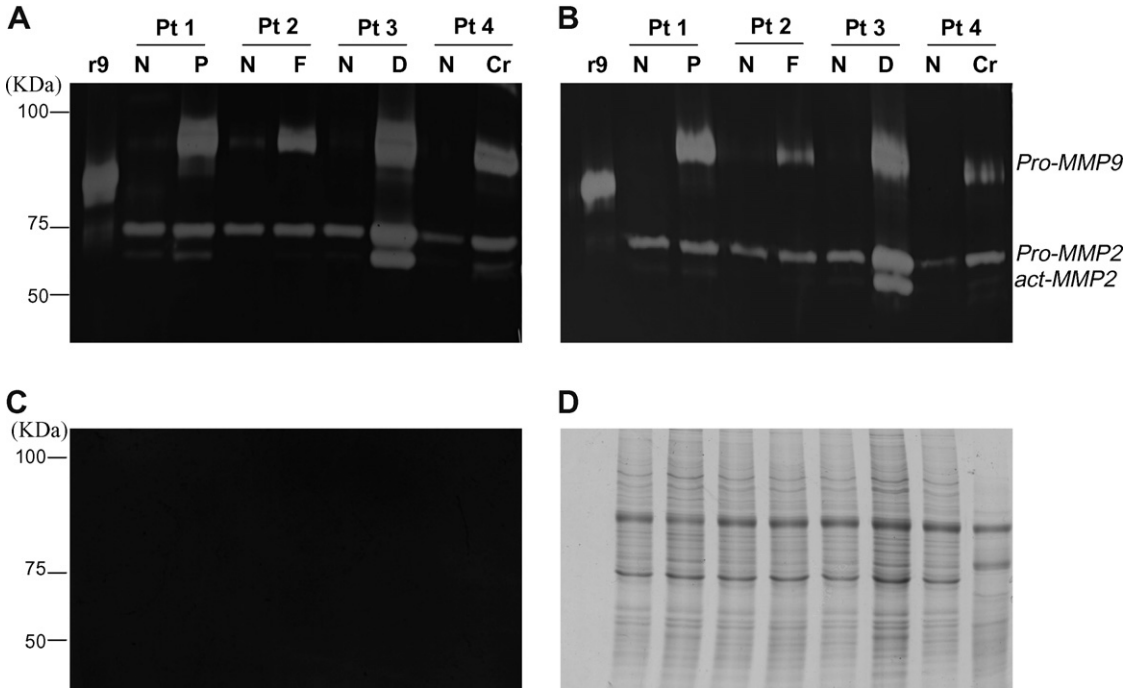

Fig. 1 – Zymographic profile of different types of adenomas. Representative samples from four patients (Pt1, Pt2, Pt3 and Pt4) were loaded at 25 μg protein per lane on gelatin-containing gels and incubated overnight in developing buffer alone (A) or suplemented with 2 μg/mL aprotinin (serine protease inhibitor) (B) and 0.1 mmol/L o-phenantroline (metalloproteinase inhibitor) (C) to confirm that the observed gelatinolytic bands were caused by MMP activity. A Coomasie blue staining was performed as a loading control (D). *Lanes N*: non-involved biopsy specimens; *lane P*: protruded adenoma; *lane F*: flat adenoma; *lane D*: depressed adenoma; *lane Cr*: CRC specimen.

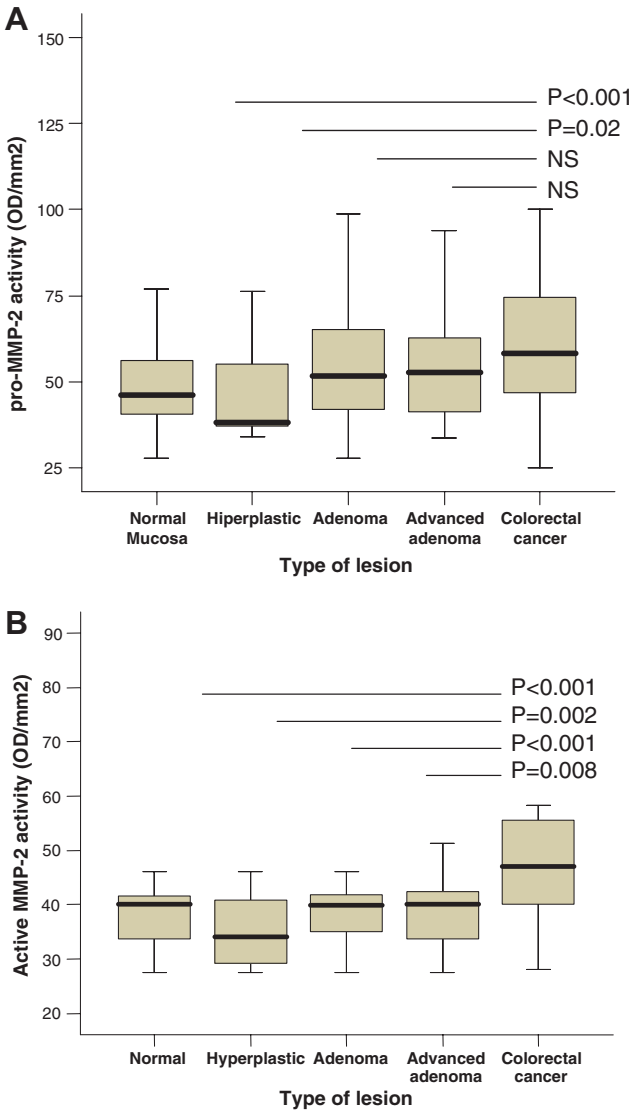

Fig. 2 – MMP-2 activity profile in neoplastic and non-neoplastic samples. The activity of pro-MMP-2 was significantly higher in CRC samples than in normal colonic mucosa or hyperplastic tissue (A). Active-MMP-2 activity was significantly higher in CRC samples than in adenomatous polyps and non-neoplastic tissues (B). The horizontal line within the box-plot corresponds to the median value and whiskers to interquartile ranges.

mm^2) than in normal mucosa (51 ± 15 OD/mm^2, $P < 0.01$), or hyperplasic polyps (47 ± 14 OD/mm^2, $P < 0.03$), but not statistically significant as compared with non-advanced (54 ± 16 OD/mm^2) or advanced adenomas (56 ± 19 OD/mm^2) (Fig. 2A). In addition, the mean activity level of active-MMP-2 in CRC samples (49 ± 16 OD/mm^2) was significantly higher than in normal mucosa (38 ± 5 OD/mm^2, $P < 0.001$) hyperplastic polyps (35 ± 6, OD/mm^2, $P = 0.002$), non-advanced (38 ± 5 OD/mm^2, $P < 0.001$) and advanced adenomas (42 ± 13 OD/mm^2, $P < 0.01$) (Fig. 2B). There were no statistically significant differences in the activity of the latent or active forms of MMP-2 between neoplastic polyps and non-neoplastic tissue, neither between hyperplastic polyps and normal mucosa.

The activity of pro-MMP-9 in neoplastic and non-neoplastic tissues is shown in Fig. 3. There was a significant and progressive activation of the latent form of MMP-9 at the different types of colorectal neoplasms. The mean level of pro-MMP-9 activity in CRC samples (96 ± 31 OD/mm^2), advanced adenomas (67 ± 17 OD/mm^2) and non-advanced adenomas (59 ± 21 OD/mm^2) was significantly higher ($p < 0.001$) than in hyperplastic polyps (41 ± 10 OD/mm^2) or normal colonic mucosal (45 ± 14 OD/mm^2) samples, respectively. In addition, CRC samples had a mean pro-MMP-9 activity significantly higher ($p < 0.001$) than in advanced or non-advanced adenomas. Finally, among neoplastic polyps, advanced adenomas presented a significantly higher ($p < 0.001$) pro-MMP-9 activity than non-advanced adenomas. Western-blot analysis confirmed the expression of pro-MMP-9 protein in colorectal adenomas, as shown by the inmunoreactive band migrating at 92 kDa (Fig. 4). As is shown in Fig. 5 a positive correlation was found between the size of the adenomas and pro-MMP-9 activity ($r = 0.37$; $p = 0.001$).

Regarding the histopathological feature of the adenomas, polyps with high grade dysplasia ($n = 11$) had a significantly higher ($p = 0.01$) pro-MMP-9 activity than those with low grade dysplasia ($n = 109$) (77 ± 10 OD/mm^2 versus 62 ± 20 OD/mm^2, respectively). By contrast, in polyps with high or low grade of dysplasia there were not statistically significant differences regarding the activity of pro-MMP2 (58 ± 33 OD/mm^{2}versus 54 ± 16 OD/mm^2, respectively) or active-MMP2 (45 ± 19 OD/mm^2 versus 39 ± 9 OD/mm^2, respectively). In addition, villous or tubulo-villous adenomas and tubular adenomas did not show statistically significant differences in the activity of

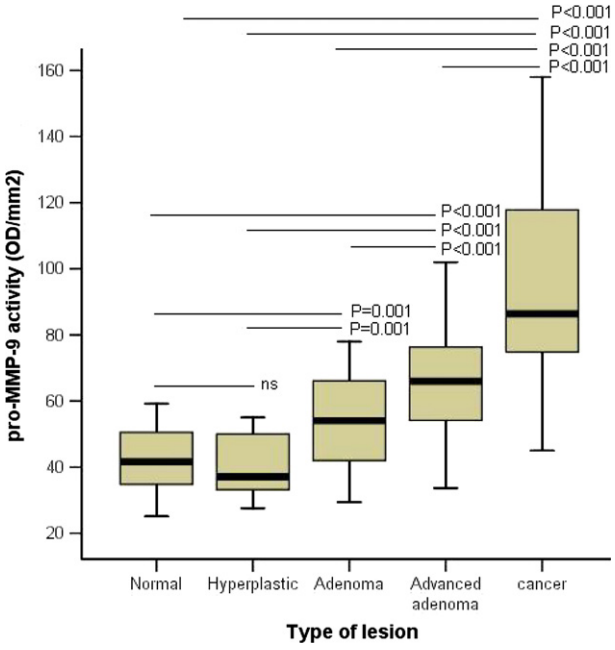

Fig. 3 – Pro-MMP-9 activity profile in neoplastic and non-neoplastic samples. There was a significant and progressive increase in the activity of the latent form of MMP-9 at the different types of colorectal neoplasms. The horizontal line within the box-plot corresponds to the median value and whiskers to interquartile ranges.

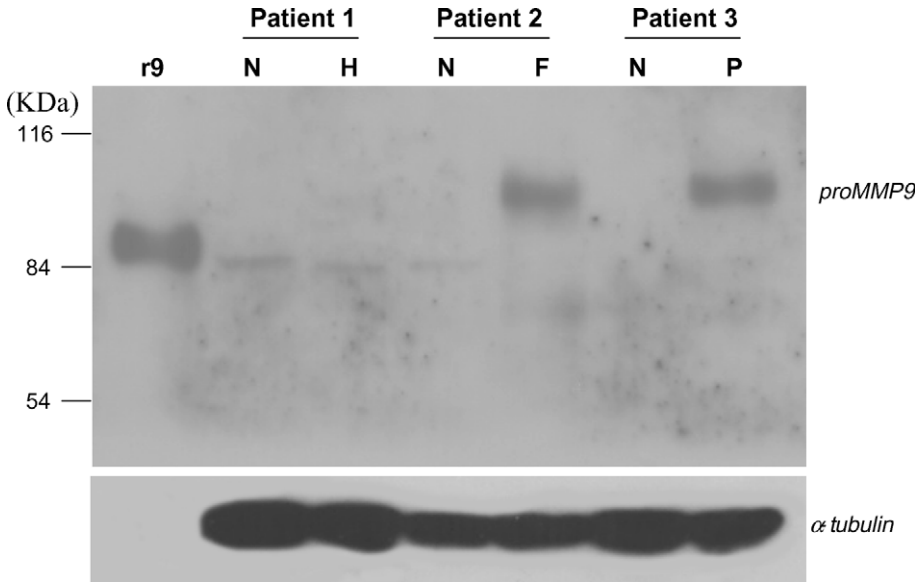

Fig. 4 – Pro-MMP-9 immunoreactivity in homogenates from colonic adenomas. Representative examples of pro-MMP-9 expression in hyperplasic polyp (patient 1, *lane H*), advanced flat adenoma (patient 2, *lane F*), and protruded adenoma (patient 3, *lane P*); Lane r9 corresponds to human recombinant MMP9. Left margin indicates relative molecular weight in Kilodaltons.

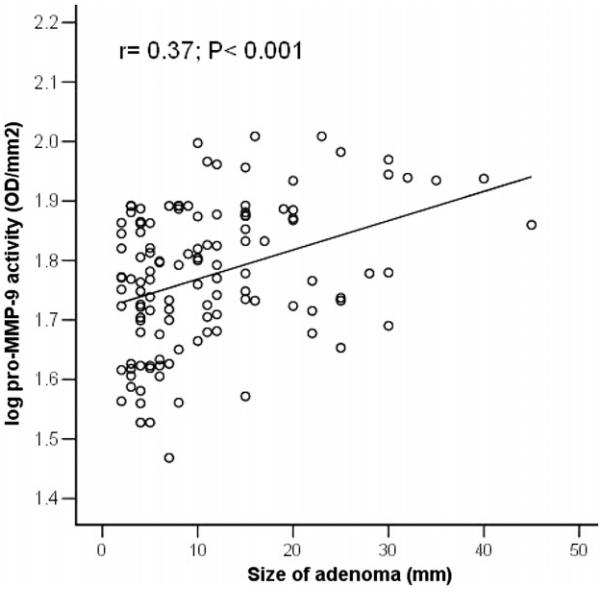

Fig. 5 – Correlation between pro-MMP-9 activity and size of adenomas. Pearson correlation showed a significant association between the size of the adenoma and pro-MMP-9 activity ($r = 0.37$, $p = 0.001$).

pro-MMP-9 (66 ± 16 OD/mm^2 versus 61 ± 20 OD/mm^2, respectively), pro-MMP-2 (55 ± 16 OD/mm^2 versus 53 ± 15 OD/mm^2, respectively) or active-MMP-2 activity (43 ± 14 OD/mm^2 versus 37 ± 5 OD/mm^2, respectively).

Finally, when gelatinase activity was evaluated according to the shape of the polyps, no statistically significant differences were found between flat and protruding adenomas, neither in pro-MMP-9 activity (62 ± 19 OD/mm^2 versus 64 ± 20 OD/mm^2, respectively) nor in pro-MMP-2 (57 ± 20 OD/mm^2 versus 53 ± 15 OD/mm^2, respectively) or in active-MMP-2 activity (42 ± 13 OD/mm^2 versus 38 ± 8 OD/mm^2 respectively). However, when the four flat-depressed lesions were assessed separately, the mean pro-MMP-9 activity was 73 ± 26 OD/mm^2, a similar level to that observed in advanced adenomas, and the active form of MMP-2 was 59 ± 21 OD/mm^2, very close to the level found in than in CRC samples.

4. Discussion

The present study shows that the activity of MMP-9 and MMP2 is markedly enhanced in advanced colorectal neoplasms. In fact, a progressive up-regulation of pro-MMP-9 activity was found in non-advanced adenomas, advanced adenomas and CRC samples as compared to hyperplasic polyps or healthy mucosa. By contrast, the latent and active forms of MMP-2 were exclusively activated in CRC samples. These findings suggest that the activation of pro-MMP-9 is an early event in the colorectal adenoma-carcinoma sequence, whereas MMP-2 activation seems to be a late phenomenon in colorectal carcinogenesis. This is in keeping with previous studies suggesting that MMP-2 is widely expressed in normal tissue being considered a house-keeping gene.[22]

Previous studies have largely investigated the role of MMP in colorectal carcinogenesis. However, most of them have focused on the behaviour and prognosis value of these enzymes on advanced CRC[10–15] rather than in the earlier steps of the adenoma-carcinoma sequence.

Colon tumours have been shown to express MMP-1, -2, -3, -8,-8,-9,-10,-11, -12 and –14.[23] In advanced CRC the expression of MMP-2 and MMP-9 has been associated with tumour invasion, hepatic metastasis and decreased survival rate.[17,24,25] However, the evidence for MMP-2 or MMP-9 up-regulation in premalignant intestinal lesions is weak. Although several reports have assessed gelatinase expression and activity in

colorectal adenomas,[10–16] results are inconsistent. Some studies have shown over-expression of these enzymes in adenomatous polyps,[10,13,25] whereas others observed very weak or no expression at all.[12,15] Parsons et al.[10] analysed by zymography the activity of the latent form of MMP-9 in a series of 53 CRC and in 15 colorectal adenomas. They found a marked up-regulation of this protease in adenomas as compared with the corresponding normal tissue. In this study, gelatinase activity was higher in carcinomas than in adenomas, but differences did not reach statistical significance. Previously, Tomita et al.[13] in an inmunohistochemistry study also observed a higher expression of MMP-9 in adenomas than in normal colonic mucosa. However, at least two recent studies[12,15] did not find any correlation between adenomatous polyps and MMP-9 expression. In analysing these conflicting results it is important to emphasise the small number of samples included in all of them, the use of different techniques for their measurement (qualitative or quantitative techniques) and the lack of characterisation of polyps according to their malignancy risk (advanced or non-advanced) or the level of dysplasia.[4,10–16]

To our knowledge no previous studies have specifically assessed the profile of gelatinases's activity in advanced and non-advanced colorectal adenomas. In the present study we found that the latent form of MMP-9 was markedly activated in advanced adenomas as compared to non-advanced adenomas, being the magnitude of that activation related to the size of the lesion. This finding suggests a role for MMP-9 in adenomatous growing. This hypothesis is supported by the fact that pro-MMP-9 activity was also up-regulated in non-advanced adenomas as compared to the healthy colonic mucosa.

The association of HGD with MMP-9 has been previously assessed in very few cases and results do not allow drawing any conclusion.[10,15] In the current study we found significant elevation of pro-MMP-9 in polyps with HGD as compared to those with low grade dysplasia. However, this finding should be interpreted with caution as only 11 polyps with HGD were included in the analysis. Regarding the possible association of these gelatinases with the morphology of colorectal adenomas, current data are confounding. We further analysed whether gelatinase activation was present in flat-colorectal adenomas. Although we did not find statistical differences in MMP-9 or MMP-2 activity between flat and protruded polyps, it was remarkable that the four flat-depressed lesions included in our study had, despite their small size (14 mm, 11 mm, 4 mm and 2 mm), up-regulation of the latent MMP-9 and active MMP-2. This is of interest as flat-depressed polyps have been shown to be associated with a higher malignancy risk than flat-elevated or protruding adenomas.[26,27] However, future studies including a large sample of flat-depressed lesions are warranted to confirm these results.

The pathogenetic mechanism by which gelatinases may be involved in the colorectal adenoma-carcinoma sequence is not well understood. It has been suggested that up-regulation of these enzymes may promote pro-angiogenic or pro-metastatic activity in solid tumours.[28] Angiogenesis is a complex and dynamic process mediated by various soluble and cell surface molecules that facilitate the interaction between tumour cells and surrounding stromal cells. MMP9 has been implicated in regulating tumour angiogenesis via a mechanism that involves vascular endothelial growth factor (VEGF) release from ECM stores.[29] A recent study confirmed that a tumour cell surface glycoprotein named extracellular matrix metalloproteinase inducer, modulates neo-angiogenesis and tumour growth throughout up-regulation of VEGF and MMP-9 among other MMP.[30] Moreover, VEGF strongly correlates with angiogenesis in colorectal adenomas and cancer.[31] Consequently, up-regulation of MMP-9 might participate in tumour growth and progression at the very early stages of colorectal carcinogenesis by means of its pro-angiogenic activity. However, gelatinases have been also associated with other potential carcinogenic mechanisms as cell differentiation, apoptosis, and immune surveillance.[28] Therefore, further investigation is warranted to clarify the mechanism by which MMP-9 may facilitate the progression from adenomatous polyps to cancer in human beings.

The present study has several limitations. First, we made histological diagnosis from biopsy specimens and not from the corresponding resected lesion, a fact that may give a discrepant diagnosis.[32] Second, although zymography is a very sensitive technique to asses gelatinase activity, we did not assess the cellular source of gelatinase production nor the specific protein location. Third, we cannot rule out the possibility that altered expression of tissue inhibitors TIMP-1 or TIMP-2 may also influence colorectal carcinogenesis, as they may be inhibitors of cancer progression by inhibiting particular MMP but may also promote cancer progression in a MMP-independent manner.[33] However, this is very unlikely as recent data support that TIMP-1 or TIMP-2 expression is a rare event in colonic adenomas or CRC.[34–36]

In summary, the present study demonstrates that up-regulation of MMP-9 is an early event in the sequence adenoma-carcinoma and might be a molecular marker for colorectal carcinogenesis. By contrast, MMP-2 up-regulation seems to be associated with advanced CRC, although it could also have a role to identify flat-depressed adenomas with invasiveness capacity. Further studies are warranted to determine the usefulness of these gelatinases as potential carcinogenic markers for colorectal adenomas. Finally, although MMP inhibitors have not been shown to be therapeutically effective in advanced gastrointestinal cancers, it would be of interest to investigate whether they have a therapeutic effect in the early stages of the sequence adenoma-carcinoma.

Conflict of interest statement

None declared.

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REFERENCES

1. Murphy G, Docherty AJ. The matrix metalloproteinases and their inhibitors. *Am J Respir Cell Mol Biol* 1992;**7**:120–5.
2. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nature Reviews* 2002;**2**:161–74.
3. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: Trials and Tribulations. *Science* 2002;**295**:2387–92.
4. Poulson R, Pignatelli M, Stetler-Stevenson WG, et al. Stromal expression of 72 kda type IV collagenase (MMP-2) and TIMP-2 mRNAs in colorectal neoplasia. *Am J Pathol* 1992;**141**:389–96.
5. Pyke C, Ralfkiaer E, Tryggvason K, et al. Messenger RNA for two type IV collagenases is located in stromal cells in human colon cancer. *Am J Pathol* 1993;**142**:359–65.
6. Zeng ZS, Cohen AM, Guillem JG. Loss of basement membrane type IV collagen is associated with increased expression of metalloproteinases 2 and 9 (MMP-2 and MMP-9) during human colorectal tumourigenesis. *Carcinogenesis* 1999;**20**:749–55.
7. Ornstein DL, Cohn KH. Balance between activation and inhibition of matriz metalloproteinase 2 (MMP-2) is altered in colorectal tumours compared to normal colonic epitelium. *Dig Dis Sci* 2002;**47**:1821–30.
8. Papadopoulou S, Scorilas A, Arnogianaki N, et al. Expression of gelatinase-A (MMP-2) in human colon cancer and normal colon mucosa. *Tumour Biol* 2001;**22**:283–9.
9. Karakiulakis G, Papanikolaou C, Jankovic SM, et al. Increased type IV collagenase degrading activity in metastases originating from primary tumours of the human colon. *Invasion metastasis* 1997;**17**:158–68.
10. Parsons SL, Watson SA, Collins HM, et al. Gelatinase (MMP-2 and MMP-9) expression in gastrointestinal malignancy. *Br J Cancer* 1998;**78**:1495–502.
11. Baker EA, Bergin FG, Leaper DJ. Matriz metalloproteinases, their tissue inhibitors and colorectal cancer staging. *Br J Surg* 2000;**87**:1215–21.
12. Liabakk NB, Talbot I, Smith RA, et al. Matrix metalloproteinase 2 (MMP-2) and matriz metalloproteinase 9 (MMP-9) type IV collagenases in colorectal cancer. *Cancer research* 1996;**56**:190–6.
13. Tomita T, Iwata K. Matriz metalloproteinases and tissue inhibitors of metalloproteinasas in colonic adenomas-carcinomas. *Dis Colon Rectum* 1996;**39**:1255–64.
14. Baker EA, Leaper DJ. Measuring gelatinase activity in colorectal cancer. *Eur J Surg Oncol*. 2002;**28**:24–9.
15. Heslin MJ, Yang J, Johnson MR, et al. Role of matrix metalloproteinases in colorectal carcinogenesis. *Ann Surg Oncol* 2001;**233**:786–92.
16. Newel KJ, Witti P, Rodgers WH, et al. Expression and localization of matrix degrading metalloproteinasas during colorectal tumourigenesis. *Mol Carcinog* 1994;**10**:199–206.
17. Zeng ZS, Huang Y, Cohen AM, Guillem JG. Prediction of colorectal cancer relapse and survival via tissue RNA levels of matrix metalloproteinase-9. *J Clin Oncol* 1996;**14**:3133–40.
18. The Japanese Research Society for Cancer of Colon and Rectum. *General rules for clinical and pathological studies on cancer of colon, rectum and anus*. 2nd ed. Tokyo: Kanehara; 1983.
19. Sawada T, Hojo K, Moriya Y. Colonoscopic management of focal and early colorectal carcinoma. *Bailliere's Clinical Gastroenterology* 1989;**3**:627–45.
20. Winawer SJ, Zauber AG. The advanced adenoma as the primary target of screening. *Gastrointest Endosc Clin N Am* 2002;**12**:1–9.
21. Fernandez-Patron C, Martinez-Cuesta MA, Salas E, et al. Differential regulation of platelet aggregation by matrix metalloproteinases-9 and -2. *Thromb Haemost* 1999;**82**:1730–5.
22. Zucker S, Vacirca J. Role of matrix metalloproteinases (MMPs) in colorectal cancer. *Cancer Metastasis Reviews* 2004;**23**:101–17.
23. Wagenaar-Miller R, Gorden L, Matrisian LM. Matrix metalloproteinases in colorectal cancer: is it worth talking about? *Cancer and Metastasis Reviews* 2004;**23**:119–35.
24. Bodey B, Bodey Jr B, Siegel SE, Kaiser HE. Prognostic significance of matrix metalloproteinase expression in colorectal carcinomas. *In Vivo* 2000;**14**:659–66.
25. Tutton MG, George ML, Eccles SA, Burton S, Swift RI, Abulafi AM. Use of plasma MMP-2 and MMP-9 levels as a surrogate for tumour expression in colorectal cancer patients. *Int J Cancer* 2003;**107**:541–50.
26. Rembacken BJ, Fujii T, Caims A, et al. Flat and depressed colonic neoplasms: a prospective study of 1000 colonoscopies in the UK. *Lancet* 2000;**355**:1211–4.
27. Fujii T, Rembacken BJ, Dixon MF, et al. Flat adenomas in the United Kingdom: Are treatable cancers being missed? *Endoscopy* 1998;**30**:437–43.
28. McCawley LJ, Matrisian LM. Matrix metalloproteinases: multifunctional contributors to tumour progression. *Mol Med Today* 2000;**6**:149–56.
29. Bergers G, Brekken R, McMahon G, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol*. 2000;**2**:737–44.
30. Tang Yi, Nakada MT, Kesavan P, et al. Extracellular matrix metalloproteinase inducer stimulates tumour angiogenesis by elevating vascular endothelial cell growth factor and matrix metalloproteinases. *Cancer Res* 2005;**65**:3193–9.
31. Kaklamanis L, Trichas M, Amarantidis K, et al. VEGF expression in the colorectal adenoma-carcinoma sequence. *Oncol Res* 2006;**15**:445–51.
32. Schlemper RJ, Kato Yo, Stolte M. Review of histological classifications of gastrointestinal epitelial neoplasia: differences in diagnosis of early carcinomas between Japanese and Western pathologists. *J Gastroenterol* 2001;**36**:445–56.
33. Joo YE, Seo KS, Kim J, et al. Role of tissue inhibitors of metalloproteinases (TIMPs) in colorectal carcinoma. *J Korean Med Sci* 1999;**14**:417–23.
34. Holten-Andersen MN, Hansen U, Brunner N, Jorgen Nielsen H, Illemann M, Schnack Nielsen. Localization of tissue inhibitor of metalloproteinases 1 (TIMP-1) in human colorectal adenoma and adenocarcinoma. *Int J Cancer* 2005;**113**:198–206.
35. Collins HM, Morris TM, Watson SA. Spectrum of matrix metalloproteinase expression in primary and metastatic colon cancer: relationship to the tissue inhibitors of metalloproteinases and membrane type-1-matrix metalloproteinase. *Br J Cancer* 2001;**84**:1664–70.
36. Masuda H, Aoki H. Host expression of matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 in normal colon tissue affects metastatic potential of colorectal cancer. *Dis Colon & Rectum* 1999;**42**:393–7.