

Beneficial Influence of Microsatellite Instability on Gelatinase-tissue Inhibitors of Metalloproteinase Balance in Colorectal Cancer

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Abstract. *Background: Colorectal cancers (CRC) with high level of microsatellite instability (MSI-H) are characterized by lower metastasis propensity and better prognosis than their stable microsatellite (MSS) counterpart. It was hypothesized that the difference in cancer progression might be related to distinct gelatinase-tissue inhibitors of metalloproteinase (TIMPs) balance in MSI-H and MSS sporadic CRC. Patients and Methods: Levels of gelatinase-A (MMP-2) and -B (MMP-9), TIMP-1 and -2 and membrane-type matrix metalloproteinase-1 (MT1-MMP) were compared in tumors and normal mucosa from patients with MSI-H and MSS CRC. Results: Active levels of MMP-2 and -9, normalized to normal mucosa, were lower in MSI-H than MSS CRC. There was a trend for higher levels of TIMP-1 and TIMP-2 within MSI-H tumors compared with MSS tumors ($p=0.08$ and $p=0.15$, respectively), while TIMP-2 amounts were significantly higher in adjacent normal tissue ($p<0.001$) in patients with MSI-H vs. MSS cancers. There was also a trend for lower MT1-MMP activity in MSI-H than in MSS CRC. Conclusion: Our data suggest that the distinct invasive and metastatic behaviors of MSI-H and MSS CRC may be related to different patterns of gelatinase secretion and regulation.*

Abbreviations: CRC: colorectal cancer; MSI: microsatellite instability; MSS: microsatellite stability; MSI-H: high level of microsatellite instability; MSI-L: low level of microsatellite instability; MMP: matrix metalloproteinase; MT1-MMP: membrane-type matrix metalloproteinase-1; TIMP: tissue inhibitor of metalloproteinase.

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Molecular analyses have shown that colorectal cancers (CRC) develop through two main genetic pathways: the chromosomal instability pathway and the microsatellite instability (MSI) pathway (1). The first pathway is characterized by allelic losses, chromosomal amplifications, translocations and aneuploidy, which are found in 85-90% of sporadic CRC. In the second pathway, CRC display widespread MSI originating from the lack of repair of DNA mismatches following DNA replication. It is well documented that CRC with high level of MSI (MSI-H) exhibit specific clinicopathological characteristics and have a better prognosis than their stable microsatellite (MSS) counterpart (2, 3). Reasons for such a difference remain unresolved; gene expression profiling, using DNA microarrays, supported the belief that the MSI-H and MSS CRC preferentially target distinct genes and signalling pathways (4).

Matrix metalloproteinases (MMPs) were ascribed a pivotal function in CRC progression, and overexpression of matrilysin (MMP-7) in MSS vs. MSI-H tumors might be associated with a higher propensity for metastasis formation (5). Among other MMPs linked to a low prognostic value in CRC are gelatinases, *i.e.*, gelatinase-A (MMP-2) and gelatinase-B (MMP-9) (6). However, unlike MMP-7, gelatinases are mainly produced by host cells, with MMP-2 being expressed by peripheral fibroblast-like cells and MMP-9 being liberated by leukocytes (7). Their overproduction is directed by diffusible mediators or direct contact between cancer and host cells (7), and the binding of gelatinases to the cancer cell plasma membrane is assumed to catalyse their activation and the formation of local proteolytic cascades required for cancer cell invasion. The activity of gelatinases is controlled by tissue inhibitors of metalloproteinases (TIMPs), which, however, were shown to display a beneficial but also a detrimental influence on cancer progression (8). The generation of active

gelatinases is directed through catalysis by other enzymes belonging to the MMP family, such as membrane-type matrix metalloproteinase-1 (MT1-MMP), which is the key activator of proMMP-2 (9). Such proteolytic crosstalk between cancer and host cells might be distinct in CRC with DNA stable or unstable microsatellites, and thus compared the levels of MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2 in tumors and normal adjacent mucosa from patients with MSI-H and MSS sporadic CRC.

Patients and Methods

Patients and tissue collection. Sixty-six sporadic CRC and their corresponding normal mucosa were obtained from patients who underwent surgery at Reims University Hospital, between 2001 and 2003. Patients who were administered preoperative radiotherapy or chemotherapy were not included. Immediately after surgery, tissue samples were harvested by a pathologist from tumor and normal colonic mucosa, at least 5 cm distant from the tumor, then snap-frozen in liquid nitrogen and stored at -80°C for subsequent measurement of MMP, TIMP and MT1-MMP levels.

Histopathological analysis of tumors. Histological analysis of formaldehyde-fixed tumors was performed on haematoxylin and eosin stained slides prepared using routine histological methods. Histopathological type, TNM stage, differentiation and tumor growth pattern were determined prospectively without knowledge of the MSI status.

Microsatellite analysis. Two blocks of formalin-fixed paraffin-embedded tissue were selected per case: one from normal colonic tissue and the other from tumor tissue. Genomic DNA was extracted using the Qia Amp Tissue Kit (Qiagen, Courtaboeuf, France). Microsatellite analysis was based on PCR amplification of 5 markers (BAT25, BAT26, D2S123, D5S346, D17S250) using fluorescence-labelled primers. They were analysed on an ABI 310 Genetic Analyser using Genescan Analysis Software (Applied Biosystem Foster City, California, USA). As recommended by the National Cancer Institute (10), tumors were classified as MSI-H, if MSI was present in at least 2 out of the 5 microsatellite markers; those with only one altered marker were classified as having low microsatellite instability (MSI-L) and those without MSI were designated as MSS. In this study, MSI-L and MSS tumors were grouped together (MSI-L/MSS) since they were reported to exhibit similar biological characteristics (11).

Determination of MMP-2 and MMP-9 activity by gelatin zymography. Tissue samples (200-400 mg) were mechanically disaggregated and homogeneous samples were extracted (50 μl per mg tissue) in buffer (50 mM Tris, 500 mM NaCl, 0.1% SDS, 0.02% NaN_3 ; pH 7.6) containing proteinase inhibitors *i.e.* 1 mM Pmsf, 5 mM EDTA, 1 mM iodoacetamide for 60 min at 4°C . After centrifugation (12,000 rpm, 4°C , 30 min) proteins were determined in supernatants using the Biorad protein assay. Tissue homogenates were stored at -80°C until use.

Extracts were diluted 1:1 with sample buffer (0.5 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 10% sodium dodecyl sulphate (SDS) and 0.01% bromophenol blue) and heated at 60°C for 15 min. After centrifugation, 10- μl aliquots containing 3 μg (tumor) or 15 μg

(normal tissue) of protein were loaded onto a 10% (w/v) Laemmli SDS-Polyacrylamide gel containing 0.1% (w/v) gelatin (type A from Porcine Skin: Sigma, Saint-Quentin-Fallavier, France) as the impregnated substrate. All gels contained either MMP-2 (0.1 ng) and/or MMP-9 (0.1 ng) as internal standards. Following electrophoresis, gels were washed twice with 2.5% TritonX-100 (w/v) pH 7.6, and incubated for 24 h in 100 mM Tris CaCl_2 , NaN_3 0.01%, pH 8.0, (Tris buffer). Zymograms were stained for 1 h with 0.1% Coomassie blue G.250 in acetic acid, methanol, water (10, 40, 50 v/v/v) solution. Proteolytic activities were visualized by clear zones within a dark blue background and quantified by determining the size of lysed area x intensity using an image analyser (Vilberd-Lourmat, France). Data were expressed as lysis areas per mg of protein.

Determination of TIMP-1 and TIMP-2 by gelatin reverse zymography. This technique presents the advantages of visualizing several TIMPs at once and of determining their total levels (free and complexed forms) (12). The overall protocol was similar to that described above for gelatin zymography except that 20 ng/ml MMP-2 were added to a 15% (w/v) Laemmli SDS-polyacrylamide gel containing 0.1% (w/v) gelatin. Following 24 h incubation of gels in Tris buffer, Coomassie staining was used to reveal TIMPs as dark blue zones against a clear background. TIMP-1 and TIMP-2 standards (Calbiochem, France) were incorporated in all gels analysed. Data are expressed as ng TIMP-1 or TIMP-2 equivalent/mg of protein.

Quantification of MT1-MMP. Active MT1-MMP was quantified using the Biotrak™ assay system (GE Healthcare GmbH, Orsay, France). In this assay, antibody-captured MT1-MMP hydrolyses the proform of a serine protease that has been engineered to be specifically activated by immobilized MT1-MMP. Therefore the serine protease activity thus generated, as determined spectrophotometrically at 405 nm using a chromogenic substrate, is directly proportional to active MT1-MMP. The sensitivity of this assay was found to be as low as 1 ng/ml. Data are expressed as ng MT1-MMP/mg of protein.

Statistical analysis. Microsatellite status was examined for association with clinicopathological parameters using Fisher's exact test. Comparisons between MMP, TIMP and MT1-MMP levels in matched tumor and normal tissues were performed using Wilcoxon sign rank tests. Comparisons between MMP, TIMP and MT1-MMP levels in tumor and normal mucosa from MSI-H and MSI-L/MSS patients were made by means of the Mann-Witney U-test. $P<0.05$ was considered statistically significant.

Results

Correlations between clinicopathological parameters and MSI status. Of the 66 specimens of CRC that were incorporated in our study, 7 (10.6%) were MSI-H. Three (5%) exhibited an MSI-L status and the remaining 56 samples ($n=56$, 84.8%) were MSS. MSI-H CRC occurred more frequently in females and were more likely located proximal to the splenic flexure although not significantly so. They appeared to be poorly differentiated as compared to MSI-L/MSS tumors ($p=0.017$) (Table I).

Levels of MMP-2 and MMP-9 in MSI-H and MSI-L/MSS colorectal cancers. Fifty-nine and 7 extracts from MSI-

Table I. Clinicopathological characteristics of MSI-H and MSI-L/MSS CRC.

	MSI-H patients (n=7)	MSI-L/MSS patients (n=59)	Statistical significance*
Age (years)			
<50	0	3	
50-70	2	25	
>70	5	31	NS
Gender			
male	3	43	
female	4	16	NS
Tumor location			
right	4	16	
left	3	43	NS
pT stage			
pT1	0	4	
pT2	1	4	
PT3	3	32	
PT4	3	19	NS
pN stage ¹			
N-	4	28	
N+	2	29	NS
metastases			
no	7	50	
yes	0	9	NS
Tumoral edge			
infiltrative	5	46	
expansive	2	13	NS
Tumor differentiation			
poor	4	8	
well	3	51	0.017

¹N stage if more than 8 lymph nodes available; *Fisher's exact test.

L/MSS and MSI-H tumors respectively were analyzed by quantitative gelatin zymography. Regardless of microsatellite status, zymograms displayed similar profiles, with lysis bands identified at 200 kDa, 135 kDa, 92 kDa, 82 kDa, 72 kDa and 62 kDa (Figure 1). The 92 and 82 kDa lytic bands corresponded to pro and active MMP-9, while the 200 and 135 kDa species might represent the homodimer and lipocalin-MMP-9 complex, respectively (13). The 72 and 62 kDa bands corresponded to pro and active MMP-2, respectively. As is apparent from Figure 1, gel loading with similar amounts of corresponding normal mucosa, whatever MSI status, led to less intense lysis bands on zymograms; therefore, MMP quantification in extracts from normal mucosa necessitated higher loading.

As shown in Table II, MSI-L/MSS tumors contained higher amounts of proMMP-9, MMP-9, proMMP-2 and MMP-2 as compared to normal tissue from the same patient ($p<0.001$). This was also true for MSI-H samples for proMMP-9 ($p=0.018$) but not for MMP-9, proMMP-2 and MMP-2 (Table II). When data were analysed comparing the

Table II. Proforms and enzyme activities of MMP-2 and MMP-9 in normal tissue and tumor from MSI-L/MMS (n=59) and MSI-H patients (n=7). Values are expressed as lysis area units per mg protein.

	Normal tissue median (interquartile range)	Tumoral tissue median (interquartile range)	Tumor/normal ratio median (interquartile range)	p-value ¹
MSI-L/MSS				
ProMMP-9	5748 (8194)	44527 (43000)	7.33 (12.3)	0.001
MMP-9	867 (1420)	3654 (4806)	2.76 (4.7)	0.001
ProMMP-2	4045 (4609)	9076 (12631)	2.34 (3.4)	0.001
MMP-2	3274 (3941)	7011 (13231)	2.72 (2.7)	0.001
MSI-H				
ProMMP-9	5883 (7205)	46171 (44840)	7.85 (9.13)	0.018
MMP-9	2273 (2314)	4770 (2909)	1.66 (1.34)	0.24
ProMMP-2	4164 (5936)	2433 (9382)	1.68 (1.62)	0.87
MMP-2	3105 (2517)	3918 (9995)	0.96 (1.84)	1

¹p-values for Wilcoxon signed rank test.

tumor/normal mucosa ratios from MSI-H and MSI-L/MSS patients, proMMP-2 and MMP-2 ratios were significantly higher in MSI-L/MSS patients. Although not reaching significance ($p=0.11$), a similar trend was apparent for the MMP-9 ratio (Figure 2).

Tumors and normal mucosa from MSI-H patients contain higher TIMP levels. TIMP-1 and TIMP-2 were identified and quantified in tissue extracts by gelatin reverse zymography (Figure 3). In several instances, the presence of an intense unidentified lysis band, running between TIMP-1 (29 kDa) and TIMP-2 (20 kDa) prevented TIMP-3 identification and interfered with TIMP-1 quantification in MSI-H CRC. Data presented in Figure 3 indicated that both MSI-L/MSS and MSI-H tumors contained significantly higher levels of TIMP-1 and TIMP-2 as compared with normal mucosa. There was also a trend for an enhancement of TIMP-1 and TIMP-2 production in MSI-H tumors compared with MSI-L/MSS tumors ($p=0.08$ and $p=0.15$, respectively). Of interest, TIMP-2 production at distance from the tumor was significantly higher in patients with MSI-H CRC than in patients with MSI-L/MSS CRC ($p<0.001$) (Figure 3).

Levels of active MT1-MMP in MSI-H and MSI-L/MSS colorectal cancer. Our data showed that, regardless of microsatellite status, normal tissues contained higher active MT1-MMP levels as compared to their tumoral counterparts, with this difference being highly significant for MSI-L/MSS CRC ($p=0.009$) (Figure 4). In addition, although significance was not attained, MSI-H tumor extracts tended to exhibit lower MT1-MMP activity than MSI-L/MSS tumors.

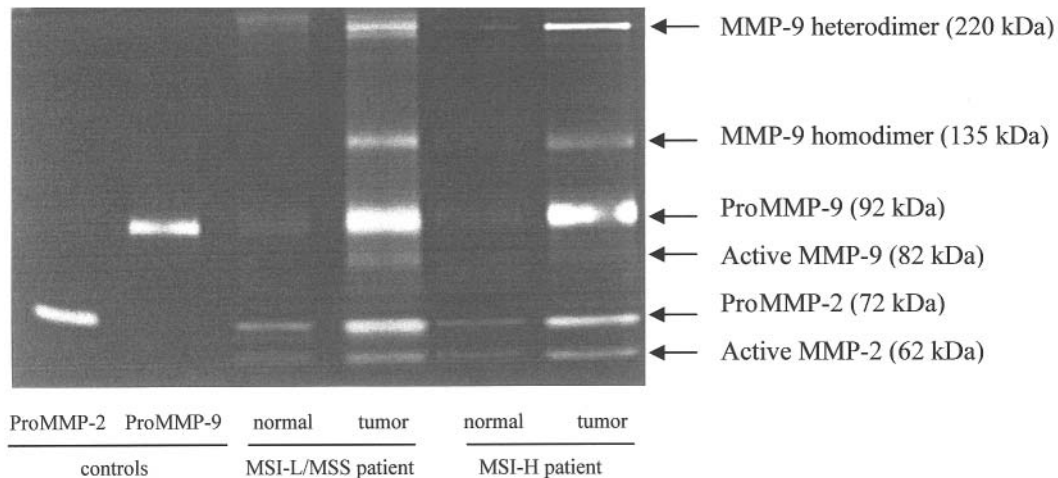


Figure 1. Gelatinolytic MMP activity detected using quantitative zymography in normal and tumoral tissues from two representative MSI-L/MSS and MSI-H patients.

Importantly, the level of active enzyme was below the limit of detection in 4 of 7 MSI-H CRC analysed (Figure 4).

Discussion

Gelatinases were consistently reported to be related to the invasive and metastatic behavior of CRC (6). MMP-9 expression in CRC was found to be associated with an increased metastasis predisposition, risk of recurrence and also decreased survival (14) and the activated forms of MMP-2 and MMP-9 were shown to be predominantly present in CRC with metastases compared with those without metastases (15).

CRC with MSI-H, representing 10-15% of overall CRC, display a clinically distinct subtype and are characterized by decreased metastasis incidence to both regional lymph nodes and distant organs (2). However, improved survival proved not to be associated with any prognostic factors. Using DNA microarrays, distinct gene expression signatures in MSI and MSS CRC have been identified, among them that for matrilysin, *i.e.*, MMP-7 (4). Moreover, genetic instability could, through mutations of genes, such as *TGFbetaR2* (16), *BAX* (17) or stromelysin (MMP-3) promoter (18), modulate the expression of several mediators influencing the host microenvironment response, particularly that of the gelatinase-TIMP balance. We used gelatin zymography to identify and quantitate levels of zymogens and active enzymes in tissue samples. Despite the low number of specimens analysed, MSI tumors, as normalized to their normal counterparts, were characterized by a significantly lower MMP-2 activity compared to MSS tumors. The presence of this active enzyme at the plasma membrane of cancer cells is of critical importance in directing the cell invasive program (9). At the pericellular environment, proMMP-2 activation proceeds *via* a complex system needing two other docking

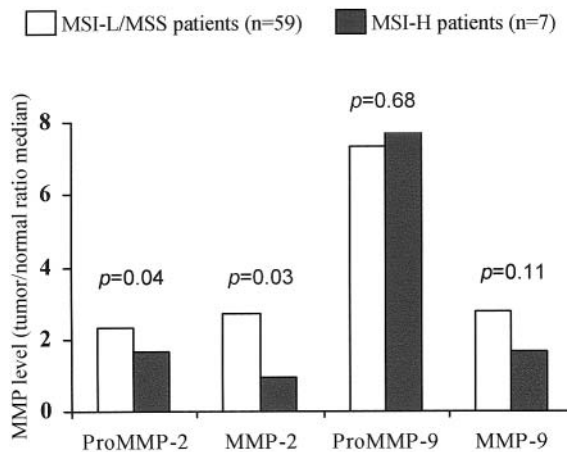


Figure 2. Levels of proMMP-2, MMP-2, proMMP-9, MMP-9 in colorectal cancer from MSI-H and MSI-L/MSS patients. Data are expressed as multiples of the MMP levels in the normal mucosa (medians).

partners *i.e.* MT1-MMP, the activator and paradoxically TIMP-2, an inhibitor for both MT1-MMP and MMP-2 (9). ProMMP-2 activation in this complex necessitates the presence of TIMP-2-free active MT1-MMP. In this intricate interplay, proMMP-2 activation depends on the respective concentrations of active MT1-MMP at the cancer cell plasma membrane and TIMP-2 mainly locally produced by surrounding fibroblasts. Of importance, MT1-MMP activity was undetected in the majority of MSI-H tumor samples we analysed; it should be emphasized that, besides acting as a proMMP-2 activator, this membrane-anchored MMP has potent activity against components of the tissue barrier and also activates $\alpha\beta_3$ integrin and induces CD44 shedding (19). In that respect, MT1-MMP was correlated with vascular invasion and the depth of invasion in CRC (20).

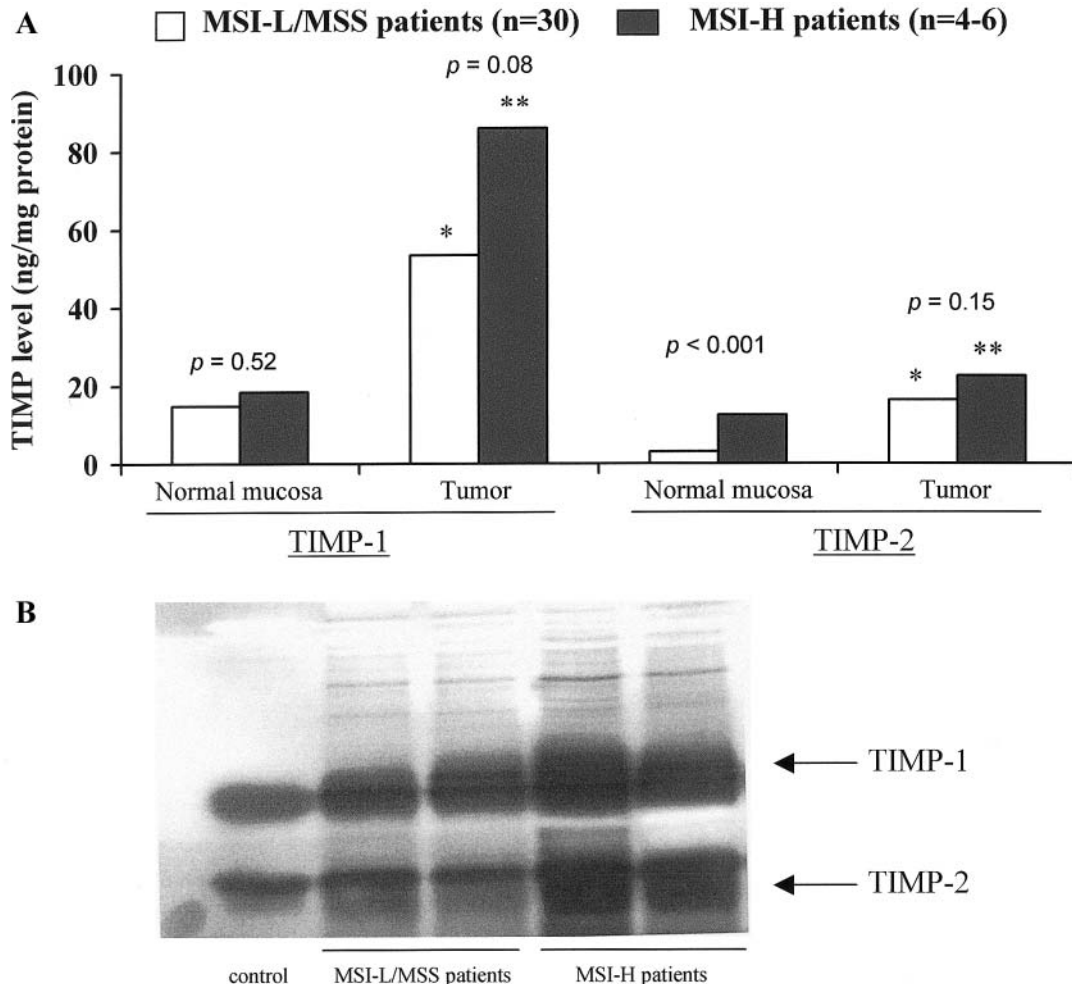


Figure 3. A) Levels of TIMP-1 and TIMP-2 in normal mucosa and colorectal cancer from MSI-H and MSI-L/MSS patients. $*p < 0.001$ and $**p = 0.068$: TIMP-1 levels in normal mucosa vs. tumor; $*p < 0.001$ and $**p < 0.046$: TIMP-2 levels in normal mucosa vs. tumor. Data are expressed as ng per mg protein (medians). B) TIMP-1 and TIMP-2 identification and quantification by gelatin reverse zymography in normal colonic tissue from 4 representative MSI-L/MSS and MSI-H patients.

MMP-9 activity also appeared to be down-regulated in MSI-H tumors as compared to MSS, although differences did not reach significance, probably related to the relatively low number of MSI-H samples in our study. This finding confirmed previous investigations by Moran *et al.* (18, 21) who showed that impairment of proMMP-9 activation in MSI-H sporadic colorectal tumors could be attributed to mutations in the promoter of MMP-3, one activator of proMMP-9, leading to lower transcriptional activity of that enzyme. ProMMP-9 might equally be activated by the MT1-MMP/MMP-2 axis (6, 9) and the lower activity of these enzymes as we reported here, together with up-regulation of TIMP-1 and TIMP-2, might also interfere with proMMP-9 activation and activity. Nevertheless, elevated levels of these MMP inhibitors does not appear to be synonymous of better prognosis and TIMP overexpression has been correlated with the progression of human colorectal cancer. This is in keeping with the

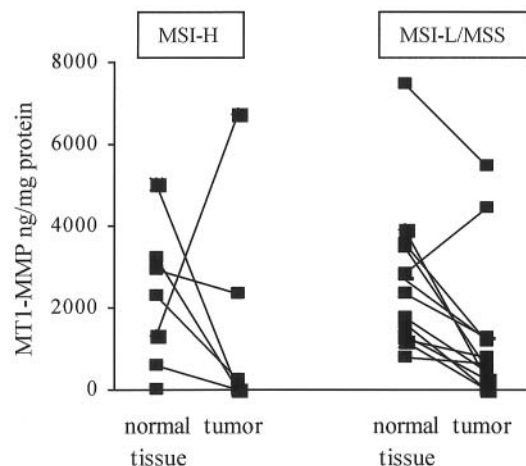


Figure 4. Levels of MT1-MMP in normal and tumoral tissue from MSI-L/MSS (n=13) and MSI-H patients (n=7). Data are expressed as ng MT1MMP/mg protein.

multifaceted characteristics of these molecules acting beneficially as MMP inhibitors and as antiangiogenic compounds but also detrimentally by participating in proMMP2 activation (TIMP-2) and displaying growth promoting activity for several cancer cell lines (8).

One striking observation in this study relies on the highly significant enhancement of TIMP-2 levels in the normal mucosa of MSI-H samples compared to MSS. Masuda and Aoki (22) pointed out that prognosis of patients with CRC is related to the level of expression of TIMP-2 mRNA in normal colon tissues. It raises the possibility that genetic/epigenetic modifications in fibroblasts due to selective pressures conferred by oncogenic stress in epithelial cancer cells might differ in CRC with or without MSI (23).

Conclusion

Our data suggest that the progression of MSI-H and MSI-L/MSS CRC may be related to different patterns of gelatinase secretion and regulation, and further illustrate the importance of the host in directing cancer progression.

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