Clinical Biochemistry 47 (2014) 1265–1271

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Expression and activity levels of matrix metalloproteinase-7 and *in situ* localization of caseinolytic activity in colorectal cancer

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ARTICLE INFO

Article history:
Received 31 January 2014
Received in revised form 31 May 2014
Accepted 2 June 2014
Available online 13 June 2014

Keywords:
Matrix metalloproteinase-7
Casein zymography
In situ casein zymography
Colorectal cancer

ABSTRACT

Objectives: Matrix metalloproteinase-7 is capable of degrading several ECM and non-ECM molecules and contributes to colorectal cancer progression and metastasis. Here, we examined the significance of MMP-7 in colorectal tumors by detecting active and latent MMP-7 levels and localization of its caseinolytic activity.

Design and methods: We investigated expression levels, localization, and proteolytic activity of MMP-7 and local caseinolytic activity in colorectal tumor and paired normal tissues by using real time PCR, casein zymography, immunohistochemistry and *in situ* casein zymography, respectively. In addition the results were compared with clinicopathological variables.

Results: Real time PCR and immunohistochemistry showed that MMP-7 expressions were higher in colorectal tumor tissues than in normal tissues. Also, mRNA expressions of MMP-7 were positively correlated with tumor and pathological stages and negatively correlated with age. Furthermore, MMP-7 mRNA expression had a sensitivity of 81.3% and a specificity of 81.2% at a cut-off value of 0.0006, making it a potential marker for diagnosis of colorectal cancer. According to casein zymography, pro- and active MMP-7 levels were also elevated in tumor tissues. In addition, we assessed local caseinolytic activity using *in situ* casein zymography. Increased immunoreactivity of MMP-7 and local caseinolytic activity were found in neoplastic cells but not in stromal cells.

Conclusion: We emphasized the significant role of MMP-7 in diagnosis and progression and/or development of colorectal cancer.

Introduction

Colorectal cancer (CRC) is the fourth common death related cancer after lung, breast and prostate cancers worldwide due to its aggressively metastatic potential [1]. Predictably, the progressive accumulation of genetic and epigenetic alterations, such as mutations in the tumor suppressor gene adenomatous polyposis coli (APC) [2], leads to transformation of normal epithelial cells to adenocarcinoma [3]. These alterations can affect the regulation of many other genes including the matrix metalloproteinase (MMP) family that contribute to colorectal tumor progression and metastasis [4].

The MMP family is comprised of more than 20 related zinc-dependent endopeptidases that are responsible for degrading and shedding several extracellular matrix (ECM) components, growth factors, receptors, cell–cell adhesion molecules, and other proteinases. Therefore, MMPs have important effects in many cellular processes including wound healing, angiogenesis, tumor progression and metastasis. The MMP family is divided into six subgroups according to their amino acid sequence, domain structures, and substrate specificity: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and macrophage elastase and other MMPs [5].

MMP-7, one of the members of matrilysins, has only a signal peptide, a prodomain and a catalytic domain. MMP-7 is secreted as 28 kDa proenzyme (pro-MMP-7) to the extracellular area and gains its activity *via* removing 9 kDa prodomain N-terminal by other proteases such as trypsin and plasmin. MMP-7 has wide range substrate specificity for ECM components such as type IV collagen, vitronectin, aggrecan, fibronectin, and various other proteoglycans [6,7]. In addition, MMP-7 has a shedding function to several non-ECM molecules including TNF-α precursor [8], FasL [9], HB-EGF [10], E-cadherin [11] and β4-integrin [12]. For this reason, it plays a crucial role in the regulation of bioactive molecules.

Due to these functions, MMP-7 is involved in apoptosis, angiogenesis, tumor growth, invasion, and metastasis. Experimental studies have

Abbreviations: CRC, colorectal cancer; APC, adenomatous polyposis coli; MMP, matrix metalloproteinase; ECM, extracellular matrix; IHC, immunohistochemistry; ADAM, a disintegrin and metalloproteinase.

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http://dx.doi.org/10.1016/j.clinbiochem.2014.06.004
0009-9120/

provided information that MMP-7 is overexpressed in 85% of human malignant colorectal tumors and its overexpression is associated with lymphatic invasion, lymph node metastasis, late Dukes' stage, and poor clinical outcome [13–16]. However, there is no clear evidence whether or not activation of MMP-7 is increased in colorectal tumors. Moreover, the localization of active MMP-7 has not yet been fully elucidated. Therefore the aim of this study was to investigate the significance of MMP-7 in colorectal tumors by detecting pro- and active MMP-7 levels and localization of its caseinolytic activity.

Material and methods

Patients and tissue samples

Thirty-seven colorectal tumor and thirty-seven paired normal tissue (taken at a site at least 10 cm from the tumor) samples were obtained from CRC patients who underwent surgical resection at the Department of General Surgery, Dokuz Eylul University School of Medicine. The protocol of this study was approved by the Ethics Committee at Dokuz Eylul University, and all patients signed consent forms of the study. For real time PCR, casein zymography and *in situ* casein zymography techniques, the samples were immediately frozen in liquid nitrogen after surgery and then stored at −80 °C. For immunohistochemistry (IHC) analysis, the tissues were fixed in formalin. The clinicopathological variables of samples are shown in Table 1 and the tumor and pathological stages were defined according to International Union Against Cancer/American Joint Committee on Cancer TNM staging system [17].

Total RNA isolation and real time PCR

Total RNA extraction was performed from tissue samples by using Trizol (Roche Applied Science, Indianapolis, IN, USA) reagent in accordance with the manufacturer's instructions and then stored −80 °C. The RNA quality was assessed by formaldehyde-denatured agarose gel electrophoresis. Isolated total RNA was reverse transcribed to complementary DNA (cDNA) with the First Strand cDNA synthesis kit according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN, USA). For real time PCR analysis, β-actin was used as a housekeeping gene and primers were chosen to amplify a section of cDNA that crossed an intron region. Real time PCR was carried out by using Roche Light Cycler System with SYBR Green I (Roche Applied Science, Indianapolis, IN, USA) and the absolute amount of MMP-7 and β-actin was determined by using a standard curve (C_T value *versus* log of amount of standard) which was generated with external standards. The results were analyzed with Light Cycler Software 4.05 (Roche Applied Science, Indianapolis, IN, USA) and presented as relative values which are the ratio of the number of copies of MMP-7 to that of β-actin.

Immunohistochemistry

The tissue sections which were dewaxed in xylene were treated with graded alcohols for rehydration. Slides were heated in 0.1 M citrate buffer (pH 6.0) in a microwave oven and then cooled to room temperature in the buffer. After washing with PBS, to restrain endogenous peroxidase activity slides were treated with Peroxidase Blocking Reagent (DakoCytomation, Hamburg, Germany) for 5 min. The slides were incubated overnight at 4 °C with monoclonal mouse antibody for MMP-7 (clone ID2) (Calbiochem Merck, Darmstadt, Germany). Subsequently, slides were incubated with Peroxidase Labeled Polymer for 30 min at room temperature. The slides were stained with 3-amino-9-ethylcarbazole and counterstained with hematoxylin. For the negative control, the sections were not treated with the primary antibodies. The sections were investigated using an Olympus BH-2 light microscope (Olympus, Tokyo, Japan). The positivity of immunoreactivity was scored for semi-quantitative evaluation, according to severity and distribution of intracytoplasmic expression in the cells [18].

Table 1
Clinicopathological characteristics of CRC patients.

Parameter	Case	
	Number	Percentage (%)
Sex		
Male	26	70.3
Female	11	29.7
Age		
≤65	21	56.8
>65	16	43.2
Neoadjuvant therapy		
Present	21	56.8
Absent	16	43.2
Tumor location		
Colon	9	24.3
Rectum	28	75.7
Tumor size		
<5 cm	29	78.4
≥5 cm	8	21.6
Tumor differentiation		
Low	29	85.3
High	5	14.7
Mucinous tumor		
Present	5	13.5
Absent	32	86.5
Metastasis		
Present	4	10.8
Absent	33	89.2
Tumor stage		
Early stage (Tis-T1–T2)	17	45.9
Late stage (T3–T4)	20	54.1
Lymph node stage		
N0	21	56.8
N1	9	24.3
N2	6	16.2
NX	1	2.7
Pathologic stage		
pE0	1	2.7
pE1	12	32.4
pE2	8	21.6
pE3	12	32.4
pE4	4	10.8
Perineural invasion		
Present	7	18.9
Absent	30	81.1
Lymphatic invasion		
Present	9	23.7
Absent	26	68.4
Venous invasion		
Present	8	78.4
Absent	29	21.6

Casein zymography

Casein zymography was performed according to Heussen and Dowdle's [19] modified method to detect active and latent MMP-7 activity levels semi-quantitatively. Total protein concentrations of tissue extracts were measured with BCA (bicinchoninic acid) assay (Thermo Scientific, Rockford, IL, USA). The levels of pro- and active MMP-7 were detected by 12% polyacrylamide gels containing 1 mg/mL β-casein. In brief, 40 μg of total protein was mixed with non-reducing sample buffer and loaded on the gel. Samples were electrophoretically separated at a constant voltage of 120 V for 2 h at +4 °C (Bio-Rad Life Science, Hercules, CA, USA) under non-denaturing conditions. To remove SDS, the gels were washed twice in 2.5% (v/v) Triton X-100 for 15 min at room temperature. Zymogram gels were then renaturated by incubating for 48 h at 37 °C in zymogram developing buffer (Invitrogen Corporation, Carlsbad, CA, USA) for digestion of the β-casein substrate. For staining and destaining, the gels were treated with 0.5% Coomassie Blue R-250 for 2 h and destaining solution (40% ethanol and 20% acetic acid in distilled water) for 15 min, respectively. Pro- and active forms of MMP-7 were defined as clear bands against a dark background of stained β-casein. Recombinant human MMP-7 protein (R&D Systems, Minneapolis, MN, USA) was