SHORT COMMUNICATION

Comparative Analysis of Matrix Metalloproteinases by Zymography in Patients With Colorectal Carcinoma

Ivana VEČURKOVSKÁ[1], Marek STUPÁK[1], Jana KAŤUCHOVÁ[2], Veronika ROŠKOVIČOVÁ[2], Jana MAŠLANKOVÁ[1]

[1]Department of Medical and Clinical Biochemistry, Faculty of Medicine, Pavol Jozef Šafárik University in Košice, Košice, Slovak Republic, [2]First Department of Surgery, Faculty of Medicine, Pavol Jozef Šafárik University in Košice, Košice, Slovak Republic

Received March 30, 2023
Accepted September 13, 2023

Summary

Zymography is an electrophoretic method in which proteins are separated in a polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE). This method is used for the detection of enzymatic activity and molecular characterization of proteins. In contrast to the standard SDS-PAGE method, a substrate is incorporated into the gel during zymography, which is subsequently cleaved by target proteases. Many studies have focused on the development and progression of inflammatory diseases affecting the gastrointestinal tract, emphasizing the role of the largest group of proteases, matrix metalloproteinases (MMPs). The most used classification of this group of enzymes (by researchers in MMP biology) is based in part on the historical evaluation of the substrate specificity of MMPs and in part on the cellular localization of MMPs. MMPs are thus classified into the groups of collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs (MT-MMPs), and others. An important group of MMPs are gelatinases which are involved in the breakdown of collagen type IV and gelatin of extracellular matrix and participate in the regulation of various physiological or pathological processes such as morphogenesis, angiogenesis, tissue repair, cirrhosis, arthritis, and metastasis. The present study's objective was to determine the amount of active MMP-9 and MMP-2 forms in tissue samples using zymography. The patient group was according to histology findings divided into the benign tumor (control) group (8 patients), and the malignant tumor group (24 patients). The respondents in the malignant tumor group were further divided according to the standard TNM classification. The results of this study confirmed that MMP-2, unlike MMP-9, can be used as a prognostic biomarker of CRC, because only the expression of active MMP-2 confirmed statistically significant differences between individual stages of CRC. Moreover, MMP-2 seems to play a more important role in higher stages of CRC. Substantial disparities in the determination of active MMPs between the observed groups support the assumption for the integration of zymography into clinical diagnostics of CRC together with molecular and other studies.

Key words

Zymography • Matrix metalloproteinases • Colorectal carcinoma

Corresponding author

J. Mašlanková, Department of Medical and Clinical Biochemistry, Faculty of Medicine, Pavol Jozef Šafárik University in Košice, Trieda SNP 1, 040 11, Košice, Slovak Republic. E-mail: jana.maslankova@upjs.sk

Metalloproteinases are a family of zinc-dependent multidomain endopeptidases. The most important class of the family is represented by matrix metalloproteinases (MMPs) [1]. MMPs are highly homologous enzymes that contain a zinc ion (Zn^{2+}) in the catalytic region and can degrade various ECM components – collagen, elastin, laminin, etc. [2]. Currently, 28 types of MMPs are known, which are categorized into 7 subclasses according to their preferred substrate or proteolytic functions. A significant step forward in clinical studies was findings that in several cancerous diseases including CRC are increased specifically MMP-2 (gelatinase A) and MMP-9 (gelatinase B), which

belong to the second subclass of MMP (gelatinases) according to their preferred substrate-gelatin [3].

MMP-2 and MMP-9 are produced in inactive (latent) pro-forms. Pro-MMP-2 (72 kDa) and pro-MMP-9 (92 kDa) are activated *via* cleaving by other proteases (plasmin, other MMPs, etc.), and so arise active MMP-2 (64 kDa) and active MMP-9 (86 kDa) [4].

The discovery that mice with a knockout gene for MMP-2 showed reduced angiogenesis and tumor growth was the first step toward confirming the significance of gelatinases in the process of angiogenesis and the development of metastases [5]. Gelatinases enable the proteolytic degradation of the vascular basal membrane and open the way for the migration of endothelial cells to form new blood vessels. MMP-2 can cleave laminin-5, creating laminin fragments that trigger migration signals in cells. The cleavage of collagen IV by MMP-2 reveals the so-called "cryptic sites", which are recognized by integrins and significantly contribute to migration stimuli [6]. MMP-9 can cleave many extracellular proteins, but also some cell surface proteins to release them from the plasma membrane. Moreover, after being activated inside of cells, MMP-9 might also cleave intracellular substrates. Based on this, MMP-9 is significantly involved in tumor progression [7]. By releasing growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and others, MMPs more and more stimulate angiogenesis during tumor formation and progression [8].

At the cellular level, epithelial-mesenchymal transition (EMT) occurs during physiological conditions (embryogenesis, organ development, tissue repair, and regeneration), but it is also considered a necessary process for oncogenesis because provides the tumor with certain aggressive properties – invasiveness and the ability to metastasize [9]. MMPs are fundamental to the EMT process since they encourage tumor cells to invade and migrate by destroying the ECM. The properties of gelatinases are crucial for both egress from metastatic cells and for entry at the site of metastasis [10].

The experimental group consisted of 32 patients who were hospitalized at the 1st surgical clinic of the UNLP in Košice and underwent surgery (biopsy/resection) because of suspected abnormalities of colon and/or rectal tissue. After the operation tissue samples were sent for histopathological examination. Based on the results, the patients were divided into two groups-patients with benign findings and patients with malignant findings. The control group consisted of 8 patients with benign findings (diverticulitis, hemorrhoids, adenoma). The malignant tumor group was further divided according to the standard TNM classification into 3 subgroups – the first stage (8 patients; T=1,2; N, M=0*), the second stage (8 patients; T=3,4; N, M=0*), and the third stage (8 patients; T=1,2,3,4; N=1,2; M=0*). All patients were classified as low-grade CRC patients. *T: Primary tumor (1: tumor affects *submucosa*, 2: tumor affects *tunica muscularis propria*, 3: tumor affects *subserosa*, 4: tumor affects other structures); N: Regional lymph nodes (0: without the presence of regional lymph nodes, 1: metastases in 1-3 regional lymph nodes, 2: metastases in 4 or more regional lymph nodes); M: Distal metastases (0: without the presence of distal metastases, 1: the presence of distal metastases).

Tissue samples were separated and cleared of blood with PBS. After 20 min of incubation on ice with commercial extraction buffer, the tissues were homogenized. Tissue homogenates were centrifuged at 18000 rpm/20 min/4 °C. The supernatant was aliquoted and stored at -80 °C. Before zymography, protein concentrations in individual samples were determined by the method of Bradford using bovine serum albumin as the standard. Tissue samples were diluted to the same concentration with 2× Laemmli Sample Buffer. Gelatin zymography took place in a 10 % polyacrylamide gel copolymerized with 1 mg/ml gelatin at 150 V. After electrophoresis, the gel was washed in 2.5 % Triton X-100 (2×30 min) and 100 mM Tris-base (2×5 min). Subsequently, the gel was incubated at 37 °C in a renaturation solution containing Zn^{2+} and Ca^{2+} ions (24 h). After incubation, the gel was stained with a 0.5 % solution of Coomassie Brilliant Blue G-250 (1 h) and subsequently destained (24 h). Bands were further processed using the ImageJ program. This program created peaks according to the intensity of individual bands and calculated areas of these peaks, which were used as a result. The GraphPad Prism (version 8.0.1.) was used to produce graphs and statistical analysis. The protein ladder was used to identify the bands, and to prove that only MMPs are present in the gel, each gel was analyzed in duplicate with the addition of PMSF.

After protein separation, we identified proteinases MMP-2 and MMP-9 on the zymograms. This study is focused only on the evaluation of their active forms (Fig. 1), because only the active forms of enzymes can carry out their intended tasks, particularly in terms of ECM degradation. Several authors have reported that MMP-9 is significantly increased in CRC patients

compared to healthy controls or patients with benign findings [11,12,13]. We confirmed these results for specifically active MMP-9 (86 kDa) using zymography, since the average of the peak areas evaluated in the ImageJ program in the control group was 315 and this average in the malignant tumor group was 489 ($p>0.05$).

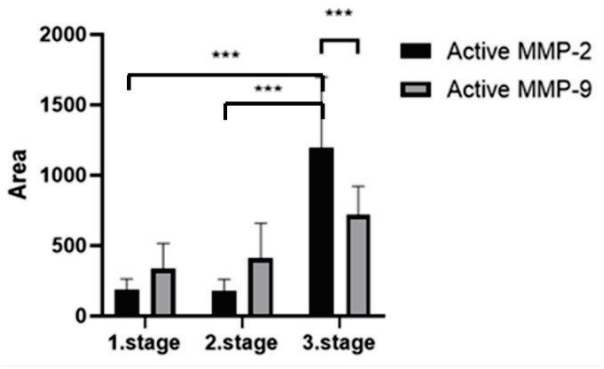

Fig. 1. The averages of active MMP-2 and MMP-9 area of peaks in clinical stages of CRC ($p<0.005$).

After dividing the patients according to the individual clinical stage of CRC, the gradual increase of active MMP-9 peak area was noticed (Fig. 2). The average of the peak areas in the first stage was 336, in the second stage was 409, and in the third stage of CRC was 721. Although the level of active MMP-9 might seem to be correlated with the stage of CRC, in this case, a statistically significant difference was not. The correlation between MMP-9 levels and stages of CRC was not confirmed by other authors [14,15].

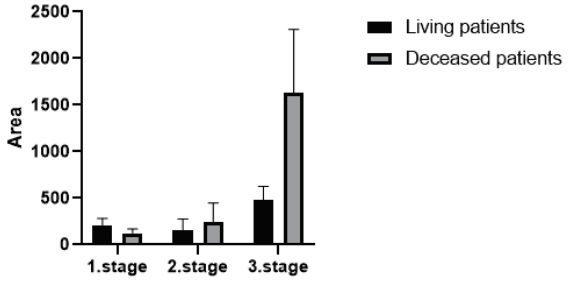

Fig. 2. The average of active MMP-2 area of peaks depending on the patient´s survival.

The average of the active MMP-2 peak areas in the control group was 370 and this average in the malignant tumor group was 521 ($p>0.05$). After dividing patients according to the clinical stage of CRC, a significant increase in the average of active MMP-2 areas peak was noticed specifically in the third stage (Fig. 1). The average of the peak areas in the first stage was 158, in the second stage was 181, and in the third stage of CRC was 1198. Kruskal-Wallis test confirmed the significant statistical difference between groups ($p=0.0048$). The most statistically significant difference was shown when comparing the second stage and third stage ($p=0.0019$), and the first stage and third stage ($p=0.007$) using the Mann-Whitney test.

Sidak´s multiple comparisons test confirmed significant statistical between the average of active MMP-2 areas peak, and the average of active MMP-9 areas peak in the third stage ($p=0.0004$) (Fig. 1). Based on this, we evaluate that in the third stage of CRC, active MMP-2 takes over the dominant role and supports tumor progression more significantly than active MMP-9.

In several studies, MMP-2 level is associated with metastasis and worse 5-year patient survival [15,16]. Our results confirm these conclusions because, in the group of patients who survived, there is no trend of a linear increase in the expression of MMP-2 in patients as it is in the group of patients who died (Fig. 2). Therefore, monitoring MMPs in patients with confirmed second stage is extremely important to capture tumor progression. We did not observe this trend of MMP-9 increase in deceased patients.

However, in the literature, we can also encounter opposing views on the prognostic potential or roles of individual MMPs during cancer, therefore the use of MMPs in clinical practice is still discussed.

In conclusion, we can state that determining the tissue active MMP-2 can play an important role in monitoring tumor progression better than active MMP-9 since statistically significant differences were confirmed when using the active MMP-2 areas of peaks in individual stages of CRC. Also, active MMP-2 is most likely more dominant in the progression of CRC compared to MMP-9. For the inclusion of MMP-2 as a prognostic marker of CRC in clinical practice, more detailed studies are needed. Substantial disparities in the determination of MMPs between the observed groups support the assumption for the integration of zymography into clinical diagnostics of CRC together with molecular and other studies. In future research, we aim to increase the research sample and focus on the evaluation of individual forms of MMPs regarding other characteristics of CRC patients to achieve more reliable results.

Conflict of Interest

There is no conflict of interest.

References

1. Zhong S, Khalil RA. A Disintegrin and Metalloproteinase (ADAM) and ADAM with thrombospondin motifs (ADAMTS) family in vascular biology and disease. Biochem Pharmacol 2019;164:188-204. https://doi.org/10.1016/j.bcp.2019.03.033
2. Bassiouni W, Ali MA, Schulz R. Multifunctional intracellular matrix metalloproteinases: implications in disease. FEBS J 2021;288:7162-7182. https://doi.org/10.1111/febs.15701
3. Lukaszewicz-Zajac M, Mroczko B. Circulating Biomarkers of Colorectal Cancer (CRC)-Their Utility in Diagnosis and Prognosis. J Clin Med 2021;10:2391. https://doi.org/10.3390/jcm10112391
4. Laronha H, Caldeira J. Structure and Function of Human Matrix Metalloproteinases. Cells 2020;9:1076. https://doi.org/10.3390/cells9051076
5. Itoh T, Tanioka M, Yoshida H, Yoshioka T, Nishimoto H, Itohara S. Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. Cancer Res 1998;58:1048-1051.
6. Quintero-Fabián S, Arreola R, Becerril-Villanueva E, Torres-Romero JC, Arana-Argáez V, Lara-Riegos J, Ramírez-Camacho MA, Alvarez-Sánchez ME. Role of Matrix Metalloproteinases in Angiogenesis and Cancer. Front Oncol 2019;9:1370. https://doi.org/10.3389/fonc.2019.01370
7. Huang H. Matrix Metalloproteinase-9 (MMP-9) as a Cancer Biomarker and MMP-9 Biosensors: Recent Advances. Sensors (Basel) 2018;18:3249. https://doi.org/10.3390/s18103249
8. Omorphos NP, Gao Ch, Tan SS, Sangha MS. Understanding angiogenesis and the role of angiogenic growth factors in the vascularisation of engineered tissues. Mol Biol Rep 2021;48:941-950. https://doi.org/10.1007/s11033-020-06108-9
9. Jayachandran J, Srinivasan H, Mani KP. Molecular mechanism involved in epithelial to mesenchymal transition. Arch Biochem Biophys 2021;710:108984. https://doi.org/10.1016/j.abb.2021.108984
10. Scheau C, Badarau A, Costache R, Caruntu C, Mihai GL, Didilescu AC, Constantin C, Neagu M. The Role of Matrix Metalloproteinases in the Epithelial-Mesenchymal Transition of Hepatocellular Carcinoma. Anal Cell Pathol 2019;2019:9423907. https://doi.org/10.1155/2019/9423907
11. Huang H. Matrix metalloproteinase-9 (MMP-9) as a Cancer Biomarker and MMP-9 Biosensors: Recent Advances. Sensors (Basel) 2018;18:3249. https://doi.org/10.3390/s18103249
12. Otero-Estévez O, De Chiara L, Rodríguez-Girondo M, Rodríguez-Berrocal FJ, Cubiella J, Castro I, Hernández V, Martínez-Zorzano VS. Serum matrix metalloproteinase-9 in colorectal cancer family-risk population screening. Sci Rep 2015;5:13030. https://doi.org/10.1038/srep13030
13. Gimeno-García AZ, Triñanes J, Quintero E, Salido E, Nicolás-Pérez D, Adrián-de-Ganzo Z, Alarcón-Fernández O, ET AL. Plasma matrix metalloproteinase 9 as an early surrogate biomarker of advanced colorectal neoplasia. Gastroenterol Hepatol 2016;39:433-441. https://doi.org/10.1016/j.gastrohep.2015.10.002
14. Koskensalo S, Hagström J, Linder N, Lundin M, Sorsa T, Louhimo J, Haglund C. Lack of MMP-9 expression is a marker for poor prognosis in Dukes' B colorectal cancer. BMC Clin Pathol 2012;12:24. https://doi.org/10.1186/1472-6890-12-24
15. Langers AM, Verspaget HW, Hawinkels LJ, Kubben FJ, van Duijn W, van der Reijden JJ, Hardwick JC, Hommes DW, Sier CF. MMP-2 and MMP-9 in normal mucosa are independently associated with outcome of colorectal cancer patients. Br J Cancer 2012;106:1495-1498. https://doi.org/10.1038/bjc.2012.80
16. Murnane MJ, Cai J, Shuja S, McAneny D, Klepeis V, Willett JB. Active MMP-2 effectively identifies the presence of colorectal cancer. Int J Cancer 2009;125:2893-2902. https://doi.org/10.1002/ijc.24682.