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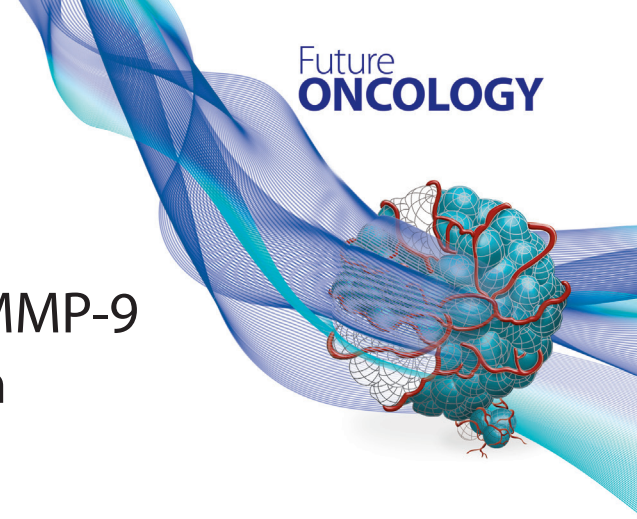

High expression of matrix metalloproteinases: MMP-2 and MMP-9 predicts poor survival outcome in colorectal carcinoma

Nada Salem[1], Ibrahim Kamal[2], Jaudah Al-Maghrabi[3], Adel Abuzenadah[1], Abdul Ali Peer-Zada[4], Yousif Qari[5], Mahmoud Al-Ahwal[5], Mohammed Al-Qahtani[1] & Abdelbaset Buhmeida*[,1]

Aim: To evaluate the expression pattern of matrix metalloproteinases (MMPs); MMP-2, MMP-7 and MMP-9 in colorectal cancer (CRC) and determine its prognostic potential. **Patients & methods:** CRC samples of 127 patients were studied. Protein expressions of MMP-2, -7 and -9 were analyzed by immunohistochemistry and association with clinicopathological variables was statistically analyzed. **Results:** Overexpressions of MMP-2 and MMP-9 correlated with poor outcome as evaluated by univariate Kaplan–Meier for disease-free survival ($p = 0.04$, $p = 0.0001$) and disease-specific survival ($p = 0.01$, $p = 0.01$), respectively. Cox analysis of MMP-2 and -9 were significant independent predictors of disease-free survival ($p = 0.006$, $p = 0.018$) and disease-specific survival ($p = 0.004$, $p = 0.049$), respectively. **Conclusion:** MMPs expression patterns provide useful prognostic information in CRC, while predicting the patients at high risk for recurrent disease.

First draft submitted: 16 August 2015; Accepted for publication: 19 November 2015; Published online: 19 January 2016

KEYWORDS
• CRC • IHC • MMPs
• prognosis

Colorectal cancer (CRC) is a crucial health problem as it makes up a significant proportion of the global cancer mortality. It accounts for 8% of all cancer-related deaths worldwide, where more than half a million people die from this disease [1]. At the level of spread, CRC is considered the third most commonly diagnosed cancer globally [2].

The prognosis of CRC is dependent on the TNM classification plus other clinical and pathological factors such as grade, level of tumor invasion, number of lymph nodes involved within tumor microenvironment and others [3]. However, despite the prognostic power of these currently employed parameters, the outcome of patients with similar stage is heterogeneous and producing different clinical outcomes. Therefore, to individualize prognosis, additional and more effective prognostic indicators at the molecular level are in high priority to differentiate and allow selection of patients with bad outcome [4].

Tumor formation is a complex, multistep process that involves the accumulation of genetic mutations in genes that regulate the pathways of cell proliferation, adhesion, differentiation and death. For the cancer to invade, epithelial cancer cells need to penetrate through the basement membrane (BM) and to disorganize the extracellular matrix which is regulated by matrix metalloproteinases (MMPs) [5]. MMPs are enormous set of zinc-containing endopeptidases, which play crucial function in the destruction of all types of extracellular matrix components. Among them are the gelatinases,

[1]Center of Excellence in Genomic Medicine Research, King Abdulaziz University, Jeddah 21589, Saudi Arabia
[2]Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah 21589, Saudi Arabia
[3]Department of Pathology, Faculty of Medicine, King Abdulaziz University, Jeddah 21589, Saudi Arabia
[4]Molecular Genetics, Pathology & Clinical Laboratory Medicine Administration, King Fahad Medical City, Riyadh 11525, Saudi Arabia
[5]Department of Medicine, Faculty of Medicine, King Abdulaziz University, Jeddah 21589, Saudi Arabia
*Author for correspondence: Tel.: +966 2640 1000, ext. 25481; Fax: +966 2695 2521; abuhmeida@kau.edu.sa

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which consist of MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa). Both are capable of degrading components of the BM, primarily type IV collagen, the foremost fundamental wall to be broken down during tumor invasiveness [6]. MMP-7, which is also known as matrilysin, is able to degrade collagens and proteoglycans and other components of BM and thus enhancing the tumor progression [7].

Growing data confirmed that formation and preservation of tumor microenvironment is primarily controlled by MMPs that mainly assist and help in the growth and angiogenesis of tumors at primary and metastatic sites, which makes them suitable candidates for predicting the patient's outcome and survival. Several studies were performed in the last decade to evaluate the roles of MMP-2, -7 and -9 in prognostication of CRC but mostly were inconsistent [8–11].

Here, we investigated immunohistochemically 127 CRC specimens of different stags (II, III and IV). Then, the association of MMP-2, -7 and MMP-9 expressions with clinicopathologic features and survival rates were analyzed by univariate and multivariate analysis. Moreover, this study, in fact, is a preliminary study and to the best of knowledge, no such study was done at our country before. We are in the process of recruiting more patients in order to evaluate the prognostic role of whole family of MMPs in CRC. Therefore, our results could serve as a new proof for the prognostic power of these proteinases in CRC.

Patients & methods

• Patients & tissue samples

The present series consists of 127 formalin-fixed and paraffin-embedded tissue samples of primary CRC obtained from patients diagnosed and treated mainly at the Departments of Surgery and Oncology, King Abdulaziz University Hospital and King Faisal Specialist Hospital and Research Center, between October 2000 and April 2011. Only specimens containing more than 80% tumor cells were used for analysis. The histopathological features of the carcinoma specimens were classified according to the TNM classification system. However, as a limitation to this study, unfortunately, two thirds (2/3) of our cohort consist of patients with CRC diagnosed at advanced stages (III and IV) and because few samples were taken from biopsies during colonoscopy, for this reason some data such as lymph node status and stage are missed. Therefore, we are in the process of recruiting more patients with different stages (early versus advanced) and will study them separately in relation to MMPs expression pattern profiles. All clinical and histopathological data of the patients were collected from the patients' case records. The key clinicopathological data of the patients are shown in **Table 1**. The samples were used according to the guidelines of the ethical committee of King Abdulaziz University Hospital.

• Immunohistochemistry

Immunohistochemistry for MMP-2 and MMP-9 was performed using automated Benchmark XT slide stainer (Ventana Medical Systems®, AZ, USA). Paraffin-embedded tissue sections were deparaffinized and pretreated using the standard Cell Conditioning buffer CC1 protocol for antigen retrieval. Sections were incubated with primary antibody MMP-2 (rabbit polyclonal anti-MMP-2; Spring Bioscience; CA, USA), MMP-7 (dilution 1: 20, Cat. no. ab5706, Abcam, Cambridge, UK) and MMP-9 (rabbit polyclonal anti-MMP-9; Spring Bioscience, CA, USA) for 32 min at 37°C, 38 min at 30°C and 42 min at 32°C, respectively. Antibody incubation was followed by application of iView™ DAB Detection Kit which includes inhibitor that contains 1.1% hydrogen peroxide solutions, biotinylated anti-mouse, peroxidase-4-conjugated streptavidin, DAB substrate and copper. The staining was ended with 4 min application of hematoxylin II, counterstained with bluing reagent for 4 min as well. Finally, sections were dehydrated in ethanol, cleared in xylene and mounted with DPX-mounting media.

• Evaluation of matrix metalloproteinases staining

The expression of MMP-2 and MMP-9 in the tumor tissues was assessed using Nikon light microscope at the magnification ×40 by A Buhmeida who was blinded to clinical data. Placental tissues were used as positive control for MMPs protein expression. The tumor cells which showed cytoplasmic staining ware graded into four categories: (0) negative, no detectable staining; (1) weak, but detectable still staining; (2) moderate, clearly positive but still weak; (3) heavy staining, intense [12]. In addition to this four-grade system, two more grading systems were used for the evaluation; two-grade

systems which classify the expression either into negative/weak and moderate/strong, or negative in one arm against all the other expressions in another arm. Comparing all the methods, negative/positive revealed the best associations of MMP-2 and -9 expressions versus the variables and disease outcome.

• Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics version 19.0 (IBM Company, NY, USA) software package. Chi-square test (X^2) was used to examine the association between MMP-2, -7 and -9 expressions with various clinicopathological characteristics. Univariate survival analysis (disease-specific survival [DSS], disease-free survival [DFS]) was based on Kaplan–Meier method, with log-rank test. DSS and DFS were calculated, based on the time from diagnosis to death (due to disease), and on the time from diagnosis to the appearance of metastatic disease or recurrence, respectively. Cox proportional hazards regression model was used to assess the value of MMP as independent predictor. The model was controlled for confounding by the following variables: age, lymph nodes status, grade (for DFS) and recurrence as additional variable (for DSS). The assumption of proportional hazards was controlled by log-minus-log survival plots. In all tests, the values $p < 0.05$ were regarded statistically.

Results

• Expression patterns of matrix metalloproteinases

The expression patterns of MMP-2 and -9 in CRC lesions are illustrated in **Figure 1**. In total 27% of all tumors were positive in cytoplasmic MMP-2 and 36% for MMP-9.

Table 1. Clinicopathological characteristics of the patients.

Characteristics	Patients, n (%)
Gender:	
– Male	63 (49.6)
– Female	55 (43.3)
– Unknown	9 (7.1)
Age (years):	
– ≤55 years	53 (41.7)
– >55 years	74 (58.3)
Histological grade:	
– Grade I (well differentiated)	19 (15)
– Grade II (moderately differentiated)	84 (66.1)
– Grade III (poorly differentiated)	8 (6.3)
– Unknown	16 (12.6)
Pathological stage:	
– Stage I	0 (0.0)
– Stage II	29 (22.8)
– Stage III	35 (27.6)
– Stage VI	33 (26)
– Unknown	30 (23.6)
Lymph node involvement:	
– No	37 (29.1)
– Yes	64 (50.4)
– Unknown	26 (20.5)
Localization:	
– Right colon	25 (19.1)
– Left colon	51 (40.2)
– Unknown	51 (40.2)
Recurrence during the follow-up:	
– Yes	44 (34.6)
– No	41 (32.3)
– Unknown	42 (33.1)
Lymphovascular invasion:	
– Yes	20 (15.7)
– No	35 (27.6)
– Unknown	72 (56.7)
Status of patient:	
–Alive	79 (62.2)
– Dead	12 (9.4)
– Unknown	36 (28.3)

MMP-2, -7 & -9 expressions in relation to clinicopathological features

Using score (low vs high), the present study **(Table 2)** revealed a significant association between MMP-2 expression and tumor localization ($p = 0.041$), recurrence ($p = 0.027$) and status at the end of follow-up period ($p = 0.018$). Accordingly, tumor arising in descending colon and rectum expresses more intense cytoplasmic MMP-2 than tumors arising in ascending and transverse colon; tumors with positive MMP-2 showed high rate of recurrence than tumors with low expression and patients with positive expression were more prone to die than MMP-2 negatively expressed patients. There was no significant association between cytoplasmic MMP-2 expression and the rest of the variables. Like MMP-2, MMP-9 shares this significant association in regard to location of tumor ($p = 0.016$) and recurrence ($p = 0.004$) along with gender ($p = 0.023$) and histological grade ($p = 0.003$). No other association was found between cytoplasmic MMP-9 expressions with clinicopathological variables. Regarding MMP-7, and by using different scoring systems mentioned above plus

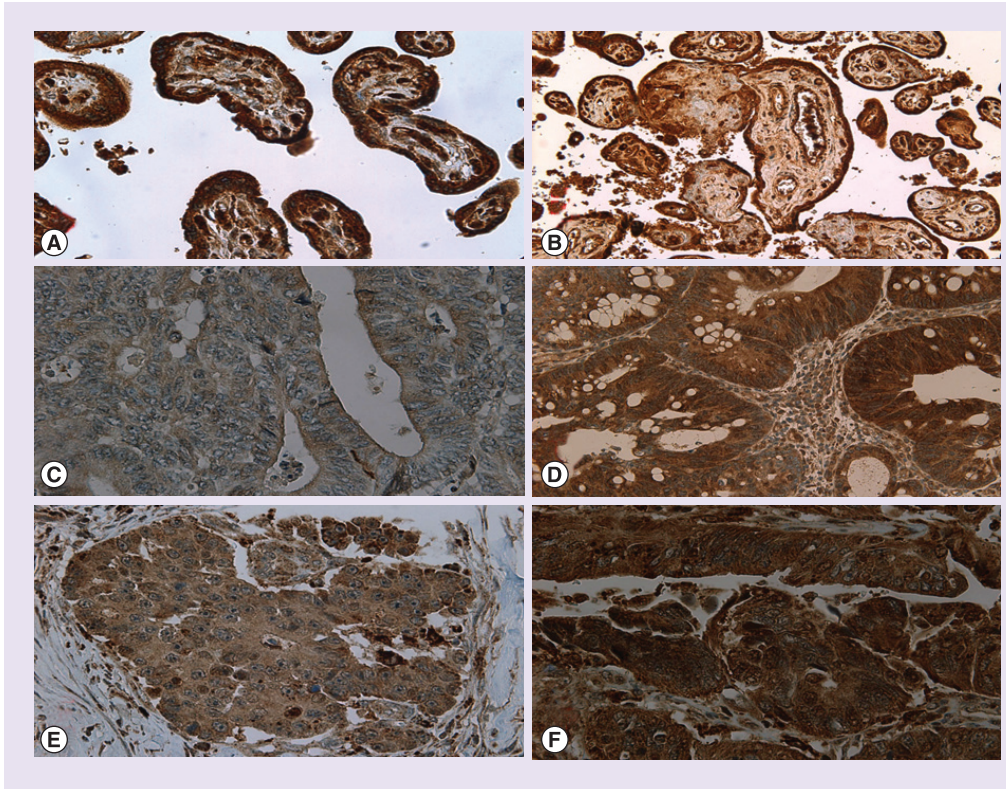

Figure 1. Expression patterns profile of matrix metalloproteinasis in colorectal cancer samples. (A & B) Placenta as a positive control for matrix metalloproteinases expression patterns. Immunohistochemical cytoplasmic of **(C)** low and **(D)** high MMP-2 expression patterns profile and cytopasmic **(E)** low and **(F)** high MMP-9 expression patterns profile, respectively.

mean and median, no correlations were found between cytoplasmic MMP-7 expression and clinicopathological variables.

Correlation of matrix metalloproteinases expression with survival outcomes

The mean overall DFS of the whole series included in MMP-2 immunostaining was 43.2 months (n = 82), and the mean DSS (operative mortality and patients died of other disease excluded) was 110 months (n = 107). In univariate (Kaplan–Meier) survival analysis, there were significant differences in which MMP-2 low expression tumors had a more favorable of both DFS (p = 0.042) and DSS (p = 0.012; **Figure 2**). Multivariate Cox regression containing age, grade, lymph node and recurrence confirmed that higher MMP-2 was the only independent factor for poor prognosis with respect to the DSS of patients (hazard ratio [HR]: 4.902; 95% CI: 1.378–17.437; p = 0.004). When the same model (omitting recurrence) was used to assess the role of MMP-2 as an independent predictor of DFS, MMP-2 also retained its significance (HR: 3.853; 95% CI: 1.481–10.024; p = 0.006) where the rest were removed from the model in stepwise backward approach.

The previous outcome also verified with MMP-9 expression in both DFS (p < 0.001) and DSS plots (p = 0.038), where the patients with low expression of MMP-9 had a longer DFS and DSS compared with those with high expression of MMP-9. The role of MMP-9 as an independent predictor of DSS was assessed in Cox analysis containing age, recurrence, lymph node status and grade variables. MMP-9 also retained its significance as an independent predictor of DSS with HR: 3.512 (95% CI: 1.008–12.239; p = 0.049; positive expression as reference). All other variables were removed from the model in stepwise backward approach. When the same model was used (omitting recurrence) to assess the role of MMP-9 as an independent predictor of DFS, only MMP-9 also proved to be an independent predictor with HR: 2.189 (95% CI: 1.145–4.188; p = 0.018; positive expression as reference). For MMP-7, the univatiate analysis revealed no significant outcome (p = 0.809) that

is based on DFS. However, for DSS the univariate analysis revealed that MMP-7 proved to be a significant predictor of poor prognosis ($p = 0.026$) in which patients with high expression patterns were likely to live shorter. Unfortunately, not enough data were available to construct the Cox regression model of both DFS and DSS.

Combinatorial approach for MMP-2 & MMP-9 with survival outcome

To estimate and compare the powerfulness of MMP-2 and MMP-9 in predicting the outcome, a combinatorial approach was applied using Kaplan–Meier analysis. **Figure 3** shows that a subgroup of positive MMP-9 and negative MMP-2 staining had significantly worse survival than those with negative MMP-9 and positive MMP-2 for both DFS ($p < 0.004$, log-rank test) and DSS ($p < 0.02$, log-rank test), respectively.

Discussion

Colorectal cancer comprises a notable proportion of the global burden in term of cancer morbidity and mortality. In Saudi Arabia, it is the most common malignant tumor for men and the second in women [13].

The CRC prognosis is relatively poor, where the outcome of the patient is defined by the extent of local and metastatic tumor spread. The estimated 5-year survival rate ranges from nearly 90% in stage I disease to <10% in patients with metastatic disease (stage IV) [14]. Currently, the most widespread method of obtaining a guide to the prognosis is achieved by histopathologic information of the tumor extent and tumor staging that is based on TNM status. However, it is widely recognized that tumors of the same pathologic stage can produce significantly different clinical courses [15]. Thus there is a need to identify more effective and clinically useful prognostic markers at genomic and proteomic levels than traditional staging system in order to detect colorectal cancer at an early curable stage and possibly to aid therapeutic decision-making [4]. The present study suggests that immunohistochemical expression for selected group of MMPs (MMP-2, MMP-7 and MMP-9) could be helpful in this detection.

In this study, we have examined the expression of MMP-2, -7 and -9 in patients diagnosed with CRC of different stages (II, III and IV). Many associations between tumor progression and these markers where identified. The most important significant finding in this cohort was that both MMP-2 and MMP-9 expressions which belong to gelatinases family were related to poor prognosis.

Overexpression of MMP-2 proved to have a prognostic power for poor outcome in Kaplan–Meier analysis in terms of DSS ($p = 0.012$) and DFS ($p = 0.042$). On Cox regression multivariate analysis, MMP-2 retained this significance and independent value in both DSS ($p = 0.004$) and DFS ($p = 0.006$) when adjusted for age, lymph node, grade and recurrence. This result is in concordance with previous studies by [16] and [17] who demonstrated the significant role of MMP-2 with shorter survival time and CRC relapse as well, in both univariate and multivariate analyses. The tendency in this study toward poor outcome has been verified in the strong association, disclosed in χ^2 test, with death ($p = 0.027$) and recurrence ($p = 0.018$). However, there were no associations with other key clinicopathological variables. This finding is similar to other studies [9,18].

Kaplan–Meier analysis of survival curves showed significantly worse DSS ($p = 0.038$) and DFS ($p = 0.0001$) of patients with high tumor MMP-9 levels, signifying that high MMP-9 protein level is indicator of worse survival outcomes for CRC patients. Furthermore, multivariate analysis attuned for age, grade, lymph node and recurrence showed that high MMP-9 expression is a sign of shorter DSS ($p = 0.049$) and DFS ($p = 0.018$) survival. These data were reported previously in multiple studies [19–21]. Expectedly, high significant correlation of MMP-9 was found with recurrence ($p = 0.004$). In contrast, our study surprisingly did not show any significant correlations between MMP-7 expression patterns and other clinicopathological features and survival outcomes except for DSS of CRC

Table 2. Relationship between the expression of matrix metalloproteinases and clinical features.

Characteristic	MMP-2 p-value	MMP-9 p-value
Gender	0.534	0.023†
Age (years)	0.831	0.428
Histological grade	0.641	0.003†
Pathological stage	0.408	0.402
Lymph node involvement	0.818	0.507
Localization	0.041†	0.016
Recurrence	0.027†	0.004†
Lymphovascular invasion	0.775	0.438
Status of patient	0.018†	0.101

†Significant associations.

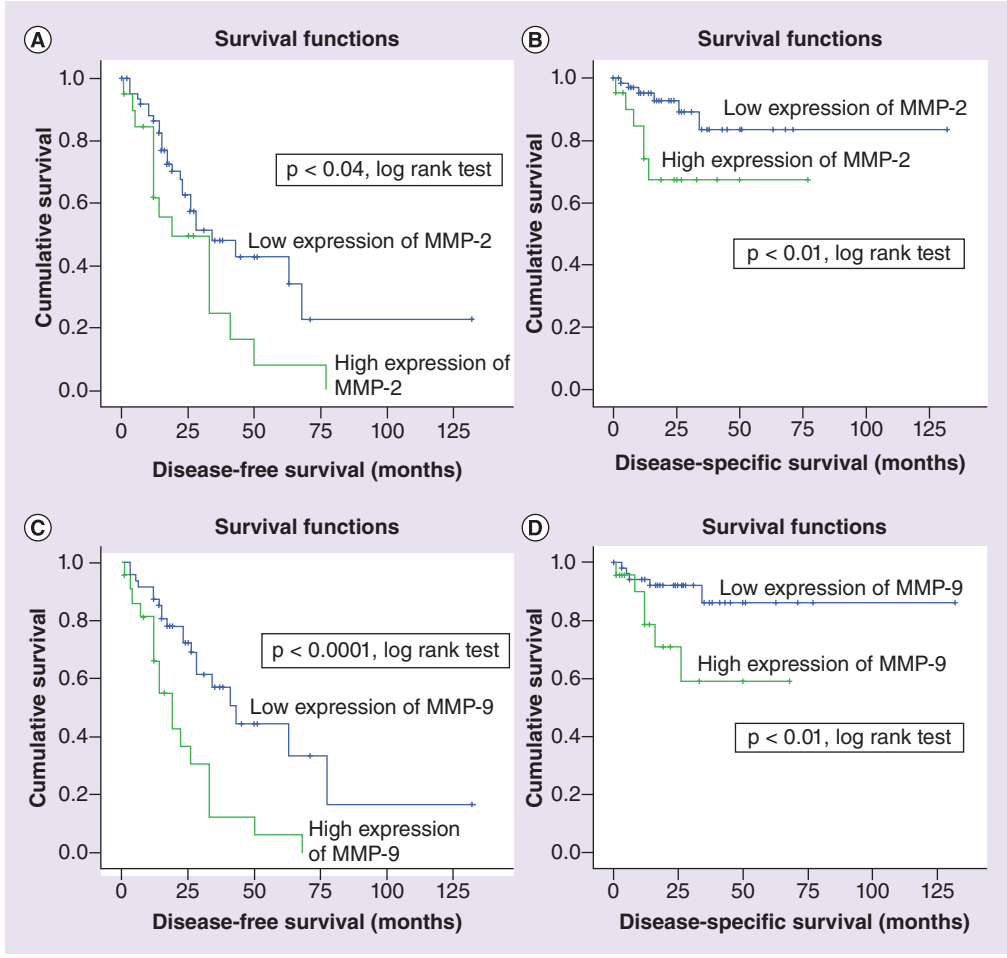

Figure 2. Comparison of MMP-2 and MMP-9 expression (low expression vs high expression) patterns profile as determinant of disease-free survival ($p < 0.04$ and $p < 0.0001$, log-rank test) and disease-specific survival ($p < 0.01$ and $p < 0.01$, log-rank test), respectively, in univariate (Kaplan–Meier) analysis.

which, in fact, is in contradictory with other studies [22,23]. This is because the prognostic value of MMP-7 in CRC remains controversial [22]. This controversy could be attributed to several factors such as sample size, the method used, scoring criteria and ethnic origin [24].

Results from the present study indicate that the expression of both MMP-2 and MMP-9 were different in relation to the site of tumor. Left-sided tumors tend to have positive MMP-2 and -9 expressions whereas right-sided tumors showed less intense expressions. This finding is in agreement with previous studies [19,25], which observed that there is difference between left and right sides of colon cancer in expression of MMP-9. However, contradicted finding by [26] suggests that MMP-2 and MMP-9 were expressed increasingly in equal amount and activity regardless of tumor location in colon and rectum. Therefore, the reason for this difference regarding the prognostic value of MMP-2 and -9 between right and left sides could be difficult to explain, but could be attributed to different molecular mechanisms and molecular phenotypes differ in carcinomas arising within these two entities.

Additional investigation to estimate the value of MMP-2 and MMP-9 in prognostication revealed that MMP-9 expression is more powerful than MMP2 in driving and predicting worse prognosis of CRC patients. **Figure 3** illustrated that the tendency of bad (worse) prognosis is almost hocked with high MMP-9 expression, and reversibly, low expression of both MMP-9 and MMP-2 boss the best prognosis in form of longer survival time.

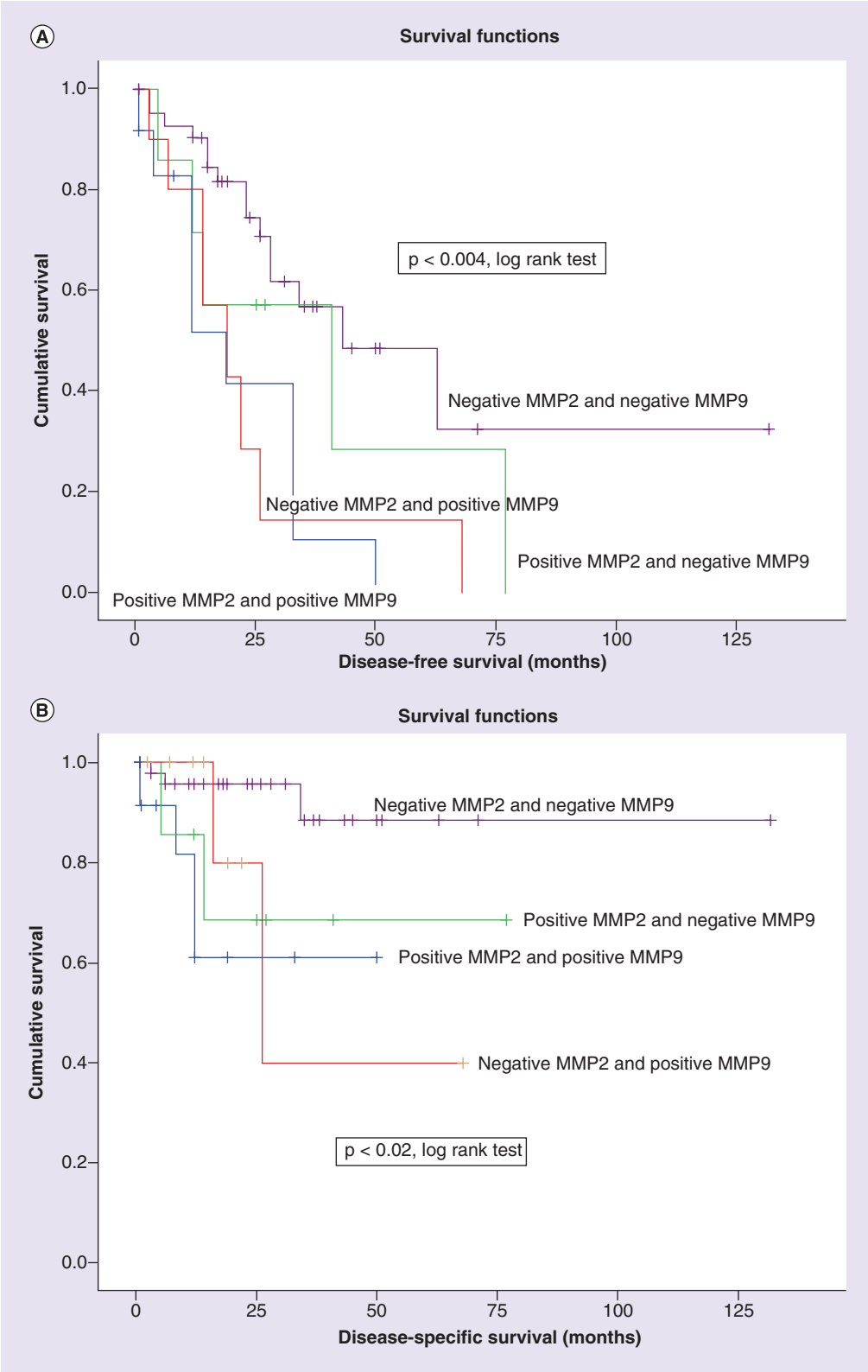

Figure 3. Combinatorial approaches of MMP-2 and MMP-9 expression patterns profiles as determinant of disease-free survival ($p < 0.004$, log-rank test) and disease-specific survival ($p < 0.02$, log-rank test) in univariate (Kaplan–Meier) analysis.

Conclusion

The preliminary results demonstrate that higher expressions of both MMP-2 and MMP-9 in CRC patients are reflecting a tendency toward recurrence and poor outcome. Therefore, it might be useful to address them along whole family of MMPs for further validation study on larger size and well-defined patients group to prove the consistency of this association. Also, further studies are needed to identify the relation between MMP-2,-7 and -9 expressions and treatment response in colorectal cancer patients. The larger proportion of patients included in this study was young and at advanced stage (III and IV) of CRC. These data emphasize the need to adopt a general population-based screening program which will reduce the CRC mortality.

Acknowledgements

The authors would like to thank the administration and laboratory staff of Center of Excellence in Genomic Medicine Research (CEGMR), King Abdulaziz University, Jeddah, for their continuous support and encouragement to perform this study.

Financial & competing interests disclosure

The research team would like to thank King Abdulaziz City for Science and Technology (KACST) for their financial support to this project under grant number (ARP-30-262). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

EXECUTIVE SUMMARY

Aim of the study

- The prognostic value of currently employed parameters, such as tumor stage and grade, is insufficient to predict the outcome of colorectal cancer (CRC) patients of similar stage but follow different clinical outcomes.
- This study aimed to evaluate the expression pattern profile of matrix metalloproteinases (MMPs); MMP-2, MMP-7 and MMP-9 in primary CRC and determine its prognostic potential.

Patients & methods

- Paraffin blocks of 127 CRC samples from different stages (II, III and IV) were retrieved. Antigen expressions of MMP-2, -7 and -9 were analyzed by immunohistochemistry and their cytoplasmic staining was evaluated using different scoring systems.
- The association of these MMPs expressions with clinic pathological variables was statistically analyzed using Chi-square test (χ^2), univariate Kaplan–Meier and multivariate analysis Cox regression.

Results

- Overexpressions of both MMP-2 and MMP-9 were significant signal of poor outcome and recurrence as evaluated by univariate for disease-free survival ($p = 0.012$, $p = 0.001$) and disease-specific survival ($p = 0.012$, $p = 0.038$).
- MMP-2 and -9 also were significant independent predictors of disease-free survival ($p = 0.006$, $p = 0.018$) and disease-specific survival as well ($p = 0.004$, $p = 0.049$) in multivariate survival analysis.
- MMP-9 expression is more powerful than MMP-2 in driving and predicting worse prognosis of CRC patients.
- MMP-7 did not show any significant correlation with patient's outcome.

Conclusion

- MMPs expression pattern seems to provide useful prognostic information in CRC, while predicting the patients at high risk for recurrent disease.
- Larger cohort and a longer follow-up with complete data are needed to fully elucidate the value of MMPs as independent predictor of disease outcome.

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