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RESEARCH

Prognostic Value of Matrix Metalloproteinase-1/ Proteinase-Activated Receptor-1 Signaling Axis in Hepatocellular Carcinoma

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Abstract Matrix metalloproteinase-1 (MMP-1) is proposed to be involved in both tumor cell invasion and metastasis. MMP-1 proteolytically activates protease activated receptor-1 (PAR-1), which also plays an important role in tumor development and progression. However, it is currently unknown whether MMP-1 activation of PAR-1 has relevance to the progression of hepatocellular carcinoma (HCC). To address this problem, we investigate the clinicopathological and prognostic value of MMP-1/PAR-1 signaling axis in HCC. Immunohistochemistry assay was used to determine the expression of MMP-1 and PAR-1 proteins in normal and HCC tissues. The correlations of MMP-1 and PAR-1 expression with clinicopathological parameters were assessed by Chi-squared test. Patient survival and their differences were determined by Kaplan-Meier method and log-rank test. Cox regression was adopted for multivariate analysis of prognostic factors. MMP-1 and PAR-1 immunoreactivities were negative or low in normal liver tissues, but high in HCC tissues. PAR-1 expression was significantly correlated with that of MMP-1 (r=0.896, p<0.0001). The overexpression of MMP-1 and PAR-1 was significantly associated with recurrence, TNM staging and portal vein invasion of HCC. Patients with high MMP-1 and PAR-1 expression had significantly poorer overall survival (OS) (both P<0.001) and disease-free survival (DFS) (both P<0.001) when compared with patients with the low expression of MMP-1 and PAR-1. On multivariate analysis, MMP-1 and PAR-1 expression patterns were found to be independent prognostic factors for OS (both P<0.001) and DFS (both P<0.001). Our results suggest for the first time that the MMP-1/PAR-1 signaling axis might be applied as a novel marker for the prediction of recurrence and metastasis potency and a significant indicator of poor prognosis for patients with HCC.

Keywords Hepatocellular carcinoma · Matrix metalloproteinase-1 · Proteinase-activated receptor-1 · Prognosis

Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths in the world [1]. This tumor develops in patients with chronic liver diseases, and its etiopathogenesis includes viral infection (hepatitis B and C), alcohol, and aflatoxin B1 consumption [2]. Although the diagnostic and surgical approaches have made great progress in recent years, patient survival remains unsatisfactory because of a high incidence of recurrence after hepatic resection or other types of loco-regional therapy [3]. The 5-year survival rate of HCC is 25–39% following surgery [4]. Considerable interest has been generated in identifying factors that influence the prognosis of HCC. Several staging systems have been developed to predict survival period after the diagnosis of HCC [5]; however, the

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results are controversial. The molecular mechanisms leading to the development and progression of HCC remain unclear and their studies may provide novel opportunities for diagnosis, prognosis, and therapeutic interventions.

The matrix metalloproteinases (MMPs), a closely related family of zinc-dependent proteolytic enzymes, are involved in the remodeling of the extracellular matrix and the proteolytic processing of bioactive molecules [6]. MMPs contribute to normal biological processes such as embryonic development and tissue repair, and also play an important role in several steps of cancer development by regulating cancer-cell growth, differentiation, apoptosis, invasion, metastasis, angiogenesis and immune surveillance [7, 8]. In particular, the interstitial collagenase (MMP-1) has been demonstrated to be associated with the invasion and poor prognosis of breast carcinoma [9], ovarian cancer [10], malignant melanoma [11], gastric cancer [12], colorectal cancer [13] and esophageal squamous cell carcinoma [14]. Several researches on the correlation of MMP-1 expression with HCC progression have been reported in recent years; however, their results are controversial. For example, Okazaki I, et al. [15] found that MMP-1 gene transcripts and protein were observed in well-differentiated cancer cells of early HCC but not in moderately or poorly differentiated cancer cells. Thus, cancer cells producing MMP-1 in early HCC may destroy the portal tract tissue adjacent to the cancer lesion and/or the fibrous band of cirrhosis. In 2004, Matsunaga Y, et al. [16] demonstrated that the expression of MMP-1 in most of the HCC tissues was equal or low compared with those in the surrounding non-tumor tissues, although mixed expression pattern was recognized in some HCC tissues. The difference of MMP-1 expression was not related with the histological differentiation of HCC and the condition of non-cancerous area. These findings suggested little association of the clinicopathological findings of HCC with the histological expression of MMP-1. However, in 2009, Altadill A, et al. [17] reported that MMP-1 is mainly expressed by stromal cells of HCC tissues. A positive correlation between MMP-1 expression and larger size tumors was found. Moreover, they also found that all HCC patients showing elevated MMP-1 expression in stromal cells presented a poor prognosis.

Protease-activated receptors (PARs) are seven-transmembrane G protein-coupled receptors that are activated by proteolytic cleavage of their extracellular domains [18]. PARs are widely expressed in vascular and extravascular tissues and are involved in responses to vascular injury and in the regulation of inflammation. Four different PARs have been identified: PAR-1, PAR-2, PAR-3 and PAR-4. In particularly, PAR-1 is activated by several proteases, including thrombin, activated protein C and MMP-1, and plays important roles in normal biologic processes [19]. PAR-1 is also an oncogene, and the signaling though it facilitates tumor invasion, angiogenesis and metastasis by inducing the expression of genes associated with cell adhesion, invasion and survival. Recent studies have been demonstrated that PAR-1 is over-expressed in several types of cancers [20–22]. However, its expression pattern in HCC is still unclear.

In the present study, to confirm whether MMP-1 activation of PAR-1 has relevance to the progression of HCC, we investigate the clinicopathological and prognostic value of MMP-1/PAR-1 signaling axis in this tumor.

Materials and Methods

Patients and Tissue Samples

This study was approved by the Research Ethics Committee of Xiangya Hospital, China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

Tissue samples were obtained from 106 patients with HCC who underwent surgical resection in Xiangya Hospital, Central South University from March, 2006 to September, 2009. The staging for each HCC was evaluated according to TNM staging guidelines. Among them, 88 were males and 18 were females, with the median age of 50 (range 32–75) years. The clinicopathological features of patients, including gender, age, background liver, viral status, tumor size, portal vein invasion, histopathological differentiation, serum AFP level, TNM staging and recurrence of HCC are summarized in Table 1. All specimens were fixed in 10% formalin, embedded in paraffin, and cut into 3-μm serial sections for immunohistochemical staining, in addition to the usual hematoxylin-eosin staining.

Immunohistochemistry Analysis

MMP-1 and PAR-1 expression was detected immunohistochemically for paraffin-embedded specimens from 106 patients with HCC. The specimens were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. The paraffin-embedded tissues were cut at 3 μm and stained following being dried on ProbeOn Plus (Fisher Scientific International, Hampton, NH, USA). Staining was done using avidin- biotin complex with a microprobe manual stainer (Fisher Scientific International). The slide to which a paraffin section was attached went through deparaffinization and hydration, and was then treated with a solution of Peroxidase-blocking reagent (Dako, Glostrup, Denmark) to exhaust endogenous peroxidase activity. It was put in citric acid solution and heated for 10 min in a microwave and then left at room temperature for 20 min to

Table 1 Correlation of MMP-1 and PAR-1 expression with clinicopathological features of HCC

Features	Cases (N)	MMP-1 (n,%)		P	PAR-1 (n,%)		P
		Low	High		Low	High	
Gender							
Male	88	25 (28.4)	63 (71.6)	0.6	28 (31.8)	60 (68.2)	0.6
Female	18	5 (27.8)	13 (72.2)		6 (33.3)	12 (66.7)	
Age (years)							
<60	70	20 (28.6)	50 (71.4)	0.6	22 (31.4)	48 (68.6)	0.6
≥60	36	10 (27.8)	26 (72.2)		12 (33.3)	24 (66.7)	
Background liver							
Chronic liver	36	17 (47.2)	19 (52.8)	0.07	19 (52.8)	17 (47.2)	0.07
Cirrhosis	70	13 (18.6)	57 (81.4)		15 (21.4)	55 (78.6)	
Viral status							
Hepatitis virus B	77	19 (24.7)	58 (75.3)	0.3	19 (24.7)	58 (75.3)	0.5
Hepatitis virus C	18	8 (44.4)	10 (55.6)		10 (55.6)	8 (44.4)	
Both B and C	3	1 (33.3)	2 (66.7)		1 (33.3)	2 (66.7)	
Non-B and non-C	8	2 (25.0)	6 (75.0)		4 (50.0)	4 (50.0)	
Tumor size							
<5 cm	57	15 (26.3)	42 (73.7)	0.5	18 (31.6)	39 (68.4)	0.6
≥5 cm	49	15 (30.6)	34 (69.4)		16 (32.7)	33 (67.3)	
Portal vein invasion							
No	76	29 (38.2)	47 (61.8)	0.001	33 (43.4)	43 (56.6)	0.001
Yes	30	1 (3.3)	29 (96.7)		1 (3.3)	29 (96.7)	
Histopathological differentiation							
Well	32	12 (37.5)	20 (62.5)	0.09	12 (37.5)	20 (62.5)	0.08
Moderate	39	13 (33.3)	26 (66.7)		17 (43.6)	22 (56.4)	
Poor	35	5 (14.3)	30 (85.7)		5 (14.3)	30 (85.7)	
Serum AFP level							
<25 ng/mL	51	19 (37.3)	32 (62.7)	0.1	22 (43.1)	29 (56.9)	0.08
≥25 ng/mL	55	11 (20.0)	44 (80.0)		12 (21.8)	43 (78.2)	
TNM stage							
I–II	36	21 (58.3)	15 (41.7)	<0.001	23 (63.9)	13 (36.1)	<0.001
III–IV	70	9 (12.9)	61 (87.1)		11 (15.7)	59 (84.3)	
Recurrence							
No	26	15 (57.7)	11 (42.3)	0.008	16 (61.5)	10 (38.5)	0.008
Yes	80	15 (18.8)	65 (81.2)		18 (22.5)	62 (77.5)	

expose antigen hidden inside the tissue due to formalin fixation, and the process was repeated three times. To inhibit non-specific antigen-antibody reactions possible in immunohistochemical staining, reaction was done using a protein blocker (Research Genetics, Huntsville, AL, USA) for 5 min and the slide was washed thoroughly with water. The slides were incubated overnight with the primary antibodies against against MMP-1 (1:1000 dilutions, Santa Cruz Biotechnology, sc-21731, Santa Cruz, CA) and PAR-1 (1:1000 dilutions, Santa Cruz Biotechnology, sc-8202, Santa Cruz, CA) at 4°C. Secondary antibodies for the detection of primary antibodies were reacted for 10 min using anti-goat IgG (Sigma, St. Louis, MO, USA) to which biotin was attached, and then washed with buffer solution and reacted with horseradish peroxidase for 10 min. Finally, the visualization signal was developed by the addition of 3,3-diaminobenzidine tetrahydrochloride (DakoCytomation) to each slide, followed by incubation for 2 min. Slides were then washed in distilled water, counterstained with hematoxylin, and dehydrated. In each immunohistochemistry run, non-tumorous liver tissues were used as control tissues and omission of the primary antibody served as negative control.

Following a hematoxylin counterstaining, immunostaining was scored by two independent experienced pathologists, who were blinded to the clinicopathological parameters and clinical outcomes of the patients. The scores of the two pathologists were compared and any discrepant scores were trained through re-examining the stainings by both pathologists to achieve a consensus score. The number of positive-staining cells showing immunoreactivity on the cell membrane and/or cytoplasm (for MMP-1) and cytoplasm (for PAR-1) in ten representative microscopic fields was counted and the percent-

age of positive cells was calculated. The frequency of MMP-1 and PAR-1 immunoreactivity in tissue sections was evaluated as negative (0) when no positive cells were observed within the tumor, weak (1) when <30% of the tumor cells were positive, moderate (2) when 30% to 60% of the tumor cells were positive, and strong (3) when >60% of tumor cells were positive. The intensity of staining was evaluated as 0, 1, 2, and 3 for no staining, weak staining, medium staining, and strong staining, respectively. Immunohistochemical scores were determined as the sum of the frequency and intensity score for tumor cells. The final score of MMP-1 and PAR-1 expression was defined as 'Low expression' if the sum of the positive score and the staining intensity score was 0–3, and 'High expression' if the sum was 4–6. In each case, at least three different areas of tumor were valuated, and the mean of the results was taken as the final expression score.

Statistical Analysis

The software of SPSS version13.0 for Windows (SPSS Inc, IL, USA) and SAS 9.1 (SAS Institute, Cary, NC) was used for statistical analysis. Continuous variables were expressed as $\overline{X} \pm s$. The Chi-square test was used to show differences of categorical variables. Spearman rank correlation test was used to analyze the correlation between the MMP-1 expression level and the PAR-1 expression level. Patient survival and their differences were determined by Kaplan–Meier method and log-rank test. Cox regression (Proportional hazard model) was adopted for multivariate analysis of prognostic factors. Differences were considered statistically significant when p was less than 0.05.

Results

Clinical Features of 106 Patients with HCC

Of the 106 tumor specimens, 32 (30.2%) were histopathologically well differentiated, 39 (36.8%) were moderately differentiated and 35 (33.0%) were poorly differentiated. Portal vein invasion of tumor cells existed in 30 (28.3%) cases of total 106 patients and did not exist in other 76 (71.7%) patients (Table 1). Up to Dec 31, 2008, 32 (30.2%) patients were alive, 74 (69.8%) patients had died of disease. Follow-up ranged from 2 to 95 months (median, 38 months).

MMP-1 and PAR-1 Expression and their Correlations with Clinicopathological Features of HCC Patients

MMP-1 and PAR-1 high expression was detected in HCC tissues from 76 (76/106, 71.7%, Fig. 1a) and 72 (72/106, 67.9%, Fig. 1b) patients in accordance with aforementioned criteria, respectively. In contrast, MMP-1 and PAR-

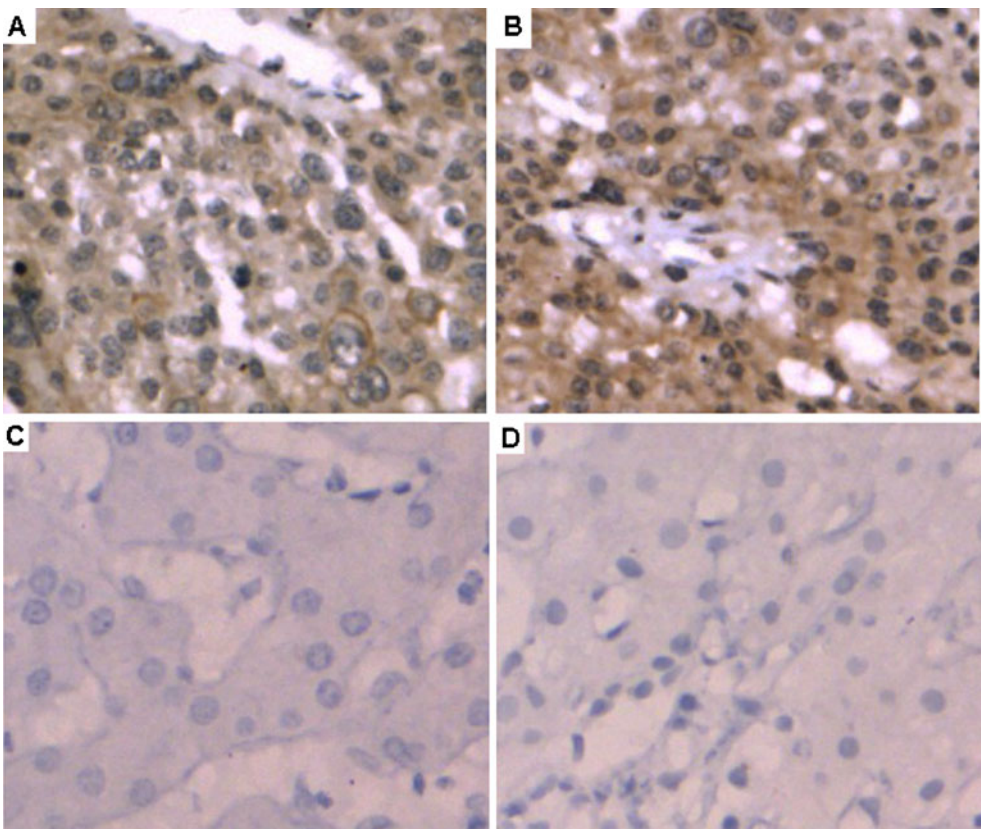

Fig. 1 Immunohistochemical staining of MMP-1 and PAR-1 proteins in HCC (**a** and **b**) and normal liver tissues (**c** and **d**) (Original magnification × 400). Staining for the two antigens is described in Materials and Methods. Intense stainings are seen in the cell membrane and/or cytoplasm (for MMP-1, **a**) and cytoplasm (for PAR-1, **b**) of tumors cells in HCC tissues. In contrast, little or no expression of MMP-1 (**c**) and PAR-1 (**d**) was observed in normal liver tissues

1 expression was absent or sporadic in normal liver tissues (Fig. 1c and d, respectively). PAR-1 expression was significantly correlated with that of MMP-1 (r=0.896, p<0.0001).

The staining scores of MMP-1 and PAR-1 were both significantly associated with recurrence (both p=0.008), TNM staging (both p<0.001) and portal vein invasion (both p=0.001) of HCC (Table 1), but not with age, gender, background liver, histopathological differentiation, viral status, tumor size and serum AFP level of tumor antigen marker (Table 1; all p>0.05). Additionally, MMP-1 and PAR-1 expression is not influenced by cirrhosis.

Prognostic Values of MMP-1 and PAR-1 Expression in HCC Tissues

Using Kaplan–Meier method and log-rank test, HCC tissues with higher staining scores of MMP-1 and PAR-1 were respectively correlated to shorter disease-free survival (DFS, Fig. 2a and c, respectively, both P<0.001) and overall survival (OS, Fig. 2b and d, respectively, both P<0.001) of patients. Besides, the survival benefits were also found in those with earlier TNM staging (P=0.01 and 0.008, respectively), lower histopathological differentiation grade (both P=0.005), absence of portal vein invasion (both P=0.01) and better background liver (P=0.009 and 0.006, respectively) for OS and DFS. Multivariate Cox regression analysis enrolling above-mentioned significant parameters revealed that MMP-1 (RR 5.263, 95%CI, 1.812–10.292, P<0.001) and PAR-1 staining scores (RR 5.021, 95%CI, 1.635–10.087, P<0.001), portal vein invasion (RR 3.698, 95%CI, 1.828–8.291, P=0.01) and TNM stage (RR 2.696, 95%CI, 1.016–7.099, P=0.03) were independent prognostic markers for OS of patients with HCC (Table 2). Meanwhile, MMP-1 (RR 5.618, 95%CI, 1.956–11.518, P<0.001) and PAR-1 staining scores (RR 5.935, 95%CI, 1.576–11.817, P<0.001) and portal vein invasion (RR 2.928, 95%CI, 1.382–5.768, P=0.006) were independent prognostic markers for DFS of patients with HCC (Table 2).

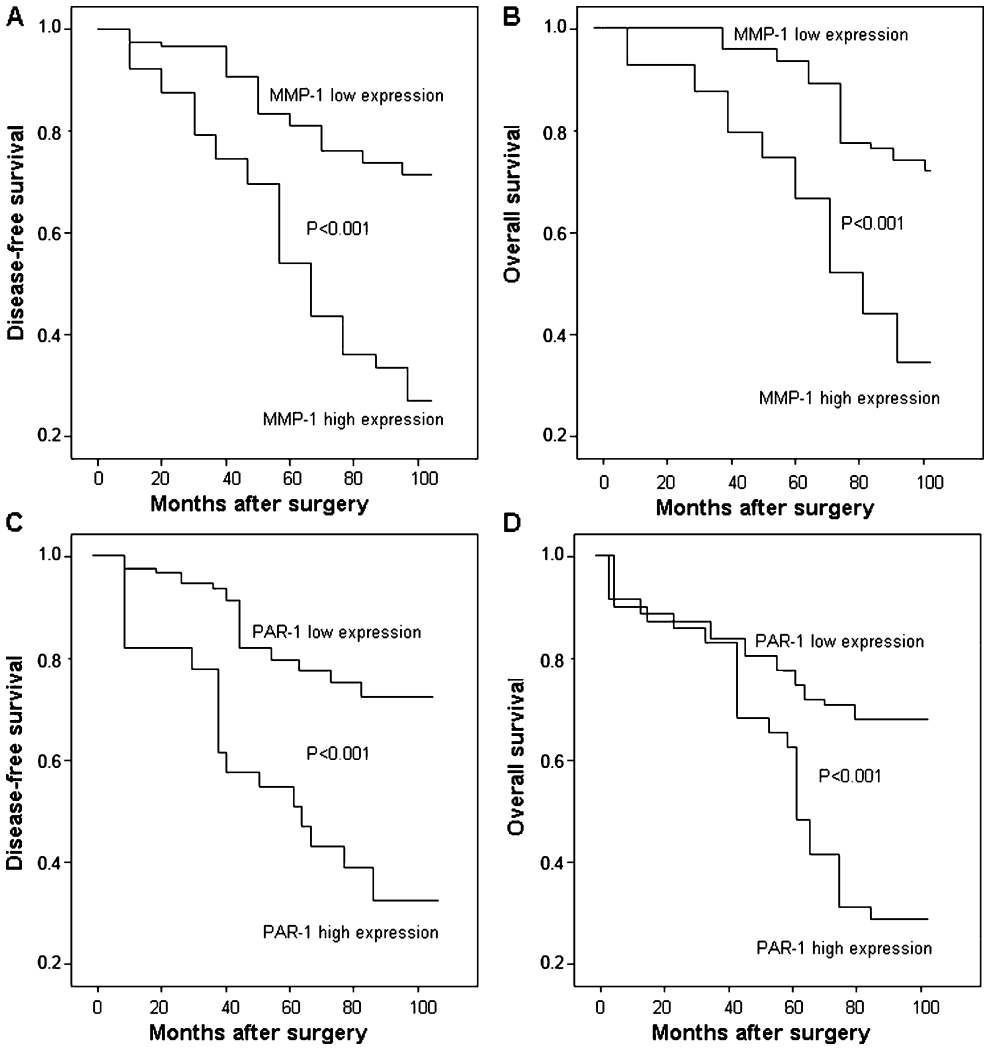

Fig. 2 Disease-free survival (**a** for MMP-1 and **c** for PAR-1) and overall survival (**b** for MMP-1 and **d** for PAR-1) curves for two groups defined by low and high expression of MMP-1 and PAR-1 in patients with HCC

Table 2 Multivariate survival analysis of OS and DFS in 106 patients with HCC

Variables	OS			DFS		
	RR	95%CI	P	RR	95%CI	P
TNM stage	2.696	1.016–7.099	0.03	1.667	0.818–3.159	0.1
Background liver	1.906	0.823–3.356	0.2	1.386	0.682–2.816	0.3
Portal vein invasion	3.698	1.828–8.291	0.01	2.928	1.382–5.768	0.006
Histopathological differentiation grade	1.369	0.982–2.313	0.2	1.479	0.903–2.138	0.2
MMP-1 expression	5.263	1.812–10.292	<0.001	5.618	1.956–11.518	<0.001
PAR-1 expression	5.021	1.635–10.087	<0.001	5.935	1.576–11.817	<0.001

Discussion

The results from our study by analyzing the expression patterns of MMP-1 and PAR-1 in 106 HCC surgical specimens using immunohistochemistry assay revealed that the high MMP-1 and PAR-1 expression levels were both associated with high TNM stage, presence of tumor recurrence and portal vein invasion, and poor survival, suggesting that MMP-1 and PAR-1 could be independent prognostic factors. This is the first study to demonstrate an association of clinicopathological features and prognostic impact of MMP-1/PAR-1 signaling axis in HCC using clinical samples in detail.

MMPs, as a family of zinc-dependent endopeptidases, are able to degrade virtually any component of the extracellular matrix. MMPs are critical for remodeling the extracellular matrix, thereby affecting cell behavior under physiologic and pathophysiologic circumstances, such as embryogenesis and cancer progression [23]. In this family, MMP-1 initiates degradation of collagen I, which is abundant in the extracellular matrix and is essential for keratinocyte migration; several authors consider that these mechanisms facilitate tumor invasion. MMP-1 expression has been described in both neoplastic and peritumoral stromal cells; however, its presence is considered more important in the zone of greatest activity corresponding to the tumor frontc [24, 25]. PAR-1, as a G protein-coupled receptor, is activated by thrombin. Apart from thrombin, several other proteases were shown to be capable of activating PAR-1 [26]. However, for a long time, the activation of tumor-expressed PAR-1 remained elusive. Only recently, Boire et al. [27] reported that MMP-1 is an additional proteolytic activator of PAR-1 promoting invasion and tumorigenesis of breast cancer cells in vitro and in vivo. The discovery of a functional MMP-1/PAR-1 interaction was inspired by the concept that host-derived MMP-1 cleaves tumor-expressed PAR-1, thus promoting the metastatic potential of cancer cells. For example, Blackburn et al. [28] reported that MMP-1/PAR-1 signaling axis promotes melanoma invasion and metastasis. Zhang et al. [29] suggested that the upregulation of MMP-1 and PAR-1 in gliomas correlates with histological malignancy grade and clinical outcome. Moreover, MMP-1 and PAR-1 immunostaining supplements the current histological grading by offering additional prognostic and predictive information.

In this study, we analyzed the correlation of MMP-1 and PAR-1 expression with clinicopathological parameters in HCC. High MMP-1 and PAR-1 expression was significantly correlated with recurrence, TNM staging and portal vein invasion of HCC, but not with age, gender, background liver, histopathological differentiation, viral status, tumor size and serum AFP level of tumor antigen marker in HCC. Moreover, patients with high MMP-1 and PAR-1 expression had significantly poorer OS and DFS when compared with patients with the low expression of MMP-1 and PAR-1. Multivariate analysis demonstrated that among the factors analyzed, with the exception of portal vein invasion, MMP-1 and PAR-1 expression levels were both independent prognostic factors for OS and DFS in patients with HCC. These results clearly demonstrated that high MMP-1 and PAR-1 expression is associated with the disease progression of HCC, and the patients with high MMP-1 and PAR-1 expression had an unfavorable clinical outcome. This raises the possibility that MMP-1 and PAR-1 may be prognostic parameters for HCC which is as or more reliable than the clinicopathological factors currently in use, and suggests the possibility to use MMP-1 and PAR-1 as targets in individualization of adjuvant therapy.

In conclusion, our results suggest that MMP-1 and PAR-1 are both overexpressed in a large proportion of patients with HCC and the high expression levels of two proteins correlated with the disease progression and poor clinical outcome in HCC. Furthermore, MMP-1/PAR-1 signaling axis proved to be a risk factor for tumor recurrence and independent molecular marker of prognosis in HCC and may become a novel target in the strategies for the prediction of tumor progression and prognosis of this disease.

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