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Overexpression of matrix metalloproteinase-12 (MMP-12) correlates with poor prognosis of hepatocellular carcinoma

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ABSTRACT

Tumour recurrence and metastasis are pressing issues of hepatocellular carcinoma (HCC) patients who receive surgical treatments. Matrix metalloproteinase-12 (MMP-12), previously identified from our animal model, is involved in tumour invasiveness of rat hepatoma. We aimed to investigate the significance and prognostic value of MMP-12 expression in human HCC.

MMP-12 mRNA level of 139 pairs of tumour and non-tumour liver tissues of HCC patients after hepatectomy were investigated by quantitative real-time RT-PCR.

MMP-12 mRNA was significantly elevated in tumour liver tissues of HCC patients compared to non-tumour and normal liver tissues. By comparing paired tumour and non-tumour liver tissues, MMP-12 mRNA was overexpressed in 58% of tumour tissue of HCC patients. Overexpression of MMP-12 mRNA was significantly correlated with presence of venous infiltration ($p = 0.004$), high serum AFP level ($p = 0.012$), early tumour recurrence ($p = 0.018$) and poor overall survival ($p = 0.02$) of HCC patients. Moreover, MMP-12 mRNA was an independent factor in predicting the 1- and 3-year overall survival of HCC patients after hepatectomy.

Our data demonstrated that MMP-12 mRNA may be a valuable prognostic marker for both overall survival and tumour recurrence of HCC patients after liver resection.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed malignancy and the third most common cause of cancer-related death in the world.[1] Surgical treatments including liver resection and liver transplantation are effective remedies for patients with HCC under stringent selection criteria. However, high incidences of tumour recurrence including intrahepatic tumour relapse and extrahepatic metastases remain a critical issue in determining the value of surgical treatments in HCC patients.[2–4] Thus, an urgent need to understand the possible molecular mechanism of HCC recurrence and metastasis as well as to identify valuable prognostic markers for stratification of HCC patients before surgical operations is warranted.

To avoid numerous confounding variables in clinical data, animal models are typically used to provide controllable and simpler circumstances for searching and studying solutions for clinical issues. However, whether findings from animal studies are clinically translatable is a concern. Our previous animal study had identified several genes including CXCL10, FOS-1, IL6, MAPK13, heat shock protein 70 (HSP70) and matrix

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metalloproteinase-12 (MMP-12) that are potentially linked to the invasiveness of rat liver tumour after liver transplantation.[5] The roles of these genes associated with tumour recurrence and metastasis in human liver cancer are indispensible for verification.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases involved in degradation of all-types of extracellular matrix components.[6] MMPs play crucial roles on cellular processes during development, remodelling process and cancers such as proliferation, angiogenesis, cell migration and apoptosis.[6–8] So far the roles of MMPs in different cancers are diverse. Several lines of evidence suggested that the roles of MMPs on tumour progression and metastasis are location-dependent; MMPs hinder tumour growth if expressed in the host while MMPs promote tumour growth if expressed in stromal cells.[8–12] Human MMP-12, also known as macrophage metalloelastase, was firstly identified from human alveolar macrophages.[13] The inactive form of human MMP-12 is a 54 kDa that is further processed by loss of both N- and C-terminal residues, into a 22 kDa mature form.[13] MMP-12 plays important roles in several diseases such as inflammatory pulmonary disease,[14] inflammatory skin disease,[15] aneurysm[16] and atherosclerosis.[17] The roles of MMP-12 in cancers are controversial. Overexpression of MMP-12 in tumours is positively associated with tumour progression and invasion in non-small cell lung cancer (NSCLC),[18–20] skin cancer,[21,22] endometrial adenocarcinoma,[23] oesophageal squamous cell carcinoma[24] and pancreatic cancer.[25] Genetic polymorphisms of MMP12 gene is also associated with poor outcome of patients with cancer diseases like breast cancer,[26] ovarian cancer,[27] oesophageal carcinoma[28] and lung cancer.[29] On the contrary, MMP-12 exhibits anti-tumour effect in cancers such as gastric cancer[30] and colorectal cancer.[31] Thus the roles of MMP-12 in different cancers require further characterisations.

Our previous animal study demonstrated that rat MMP-12 gene may be positively linked to tumour progression and invasion of rat hepatoma.[5] However, a study using clinical samples from 40 Japanese HCC patients demonstrated that overexpression of MMP-12 mRNA is significantly associated with good overall survival of HCC patients after hepatectomy.[32] The expression of MMP-12 gene in these patients is positively correlated with angiostatin production and hypovascular tumours.[32,33] In fact, risk factors of hepatocarcinogenesis between Japan and Hong Kong are different.[34] In this study, we applied a quantitative method to investigate the clinical significance of MMP-12 expression in HCC patients of our centre.

2. Methods and materials

2.1. Clinical samples

One hundred and thirty-nine HCC patients undergone liver resection between October 1999 and October 2007 were recruited from Department of Surgery, Queen Marry Hospital, the University of Hong Kong, contributing to 139 pairs of RNA samples from tumour tissues and adjacent non-tumour tissues. There were 113 men and 26 women. The age of the patients ranged from 22 to 81 years, with a median age of 55 years. One-hundred and twenty-one HCC patients (87.1%) were positive for hepatitis B surface antigen, whereas only 6 (4.3%) were positive for hepatitis C virus antibody. Ten normal liver tissues were recruited from living donors at the same hospital and examined to be free of liver diseases and hepatitis B infection. The follow-up duration for the HCC patients ranged from July 2001 to October 2010. The study was approved by the Ethics Committee of the University of Hong Kong.

2.2. Reverse transcription-PCR (RT-PCR)

Total RNA from liver tissues and cell lines were extracted by TRIZol regent according to the manufacturer's instruction (Invitrogen). Each cDNA was synthesised from 1 μg of total RNA using the High capacity cDNA Kit (Applied Biosystems) at 25 °C for 5 min and at 37 °C for 2 h subsequently. PCR reaction was performed using the Taq PCR Kit (Promega) under the following PCR cycles: 95 °C for 5 min, 35 cycles at 95 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min. For internal control 18S, 30 PCR cycles were used. Primers for MMP-12 are: sense 5′-GCT TGC CAA ATC CTG ACA-3′ and antisense 5′-CCT TCA GCC AGA AGA ACC-3′. Primers for 18S ribosomal RNA are: sense 5′-CTC TTA GCT GAG TGT CCC GC-3′ and antisense 5′-CTG ATC GTC TTC GAA CCT CC-3′.

2.3. Quantitative real-time RT-PCR

Each 1 μg of total RNA was used to synthesise cDNA using the High capacity cDNA Kit (Applied Biosystems). PCR analysis of each of the target gene was carried out in the following PCR mixture: 1 μl of cDNA, 10 μl of 2× Power SYBR Green PCR Master Mix (Applied Biosystems), 0.1 μl of 10 mM forward primer, 0.1 μl of reverse primer and 8.8 μl of distilled water. Real-time PCR was carried out in a 7700 Sequence Detection Instrument (Applied Biosystems) using the following thermal cycling profile: 95 °C 1 min, followed by 40 cycles of amplification (95 °C 15 s, 60 °C 4 min). Quantitative RT-PCR was performed in at least triplicates and repeated once for every sample. Threshold cycle (Ct) value for each sample was determined by the preset parameters of the instrument. Analysis of dissociation curve for each pair of primers was conducted to examine the specificity of each PCR product. The relative expression level of MMP-12 mRNA for each sample was calculated as: $\Delta\Delta Ct(\text{sample}) = \Delta Ct(\text{calibrator}) - \Delta Ct(\text{sample})$, where $\Delta Ct(\text{calibrator})$ of MMP-12 mRNA = Ct(calibrator) of MMP-12 – Ct(calibrator) of 18S RNA; $\Delta Ct(\text{sample})$ = Ct(sample) of MMP-12 mRNA – Ct(sample) of 18S RNA. The calibrator was defined as the sample with the highest Ct value of MMP-12 mRNA (sample with the lowest expression level of MMP-12 mRNA) among all samples. The value of Ct difference is equal to 2^n-fold difference.

2.4. Statistical analysis

The MMP-12 mRNA level among normal liver tissues and tumour as well as non-tumour liver tissues of HCC patients were analysed by Prism Version 5.01 (Graphpad). Unpaired two-tailed *t*-test was employed to analysed the correlation of MMP-12 mRNA (or $\Delta\Delta Ct(\text{sample})$) among these groups.

To investigate the expression significance of MMP-12 mRNA in HCC patients, the difference of MMP-12 mRNA level between tumour and non-tumour tissues for each HCC patient was firstly determined as: ΔΔΔCt(patient) = ΔΔCt(tumour) – ΔΔCt(non-tumour). Statistical analysis of clinical parameters was carried out using SPSS 16 for Windows (SPSS Inc.). Receiver Operating Characteristic (ROC) curve was generated to analyse the sensitivity and 1-specificity of ΔΔΔCt(patient) of MMP-12 mRNA to predict 1-year overall survival of HCC patients after hepatectomy. Youden index was used to determine the optimal cutoff point of ΔΔΔCt(patient) for predicting 1-year overall survival. The value of ΔΔΔCt(patient) equal to or higher than the cutoff point was defined as MMP-12 overexpression while the value of ΔΔΔCt(patient) less than the cutoff point was determined as MMP-12 non-overexpression. The association of MMP-12 mRNA expression (overexpression or non-overexpression) and clinicopathological parameters including sex, age, pTNM staging, venous infiltration, encapsulation, tumour size, serum alpha-fetoprotein level and hepatitis B surface antigen was analysed by Chi-square test. The prognostic value using MMP-12 mRNA for predicting the overall survival and disease-free survival of HCC patients after hepatic resection was calculated by Kaplan–Meier analysis with the log-rank test. For Kaplan–Meier analysis of disease-free survival, HCC patients who were under the category of hospital mortality were excluded. Cox proportional hazard regression model was performed with univariable and multivariable analyses to test factors that were significantly associated with the overall survival of the HCC patients. Logistic regression analysis was also performed to compare those factors for predicting 1-, 3- and 5-year overall survival of the HCC patients after liver resection. *P* value <0.05 was considered to be statistically significant.

3. Results

3.1. *Expression of MMP-12 mRNA in HCC patients and normal donors*

The expression level of MMP-12 in 18 pairs of tumour and non-tumour liver tissues of HCC patients were preliminarily examined by traditional RT-PCR analysis. MMP-12 transcript was found to be overexpressed in 14 tumour tissues (77.78%) compared to non-tumour tissues of HCC patients (Fig. 1A). There was no MMP-12 mRNA detected in the recruited 10 normal liver tissues (Fig. 1B).

To further examine the mRNA level of MMP-12 in more clinical samples and in a quantitative way, real-time RT-PCR was employed to examine the MMP-12 mRNA in 139 pairs of tumour and non-tumour tissues of HCC patients and 10 normal liver tissues. The average ΔΔCt value of MMP-12 among normal liver tissues, non-tumour liver tissues and tumour live tissues were 1.32, 4.89 and 8.60, respectively (Fig. 2). Analysis from unpaired two-tailed *t*-test showed that the expression level of MMP-12 mRNA was statistically significant between normal and non-tumour tissues ($P < 0.0001$, $t = 4.573$, $df = 147$), between normal and tumour tissues ($P < 0.0001$, $t = 5.493$, $df = 147$) as well as between non-tumour and tumour tissues ($P < 0.0001$, $t = 9.080$, $df = 276$, Fig. 2).

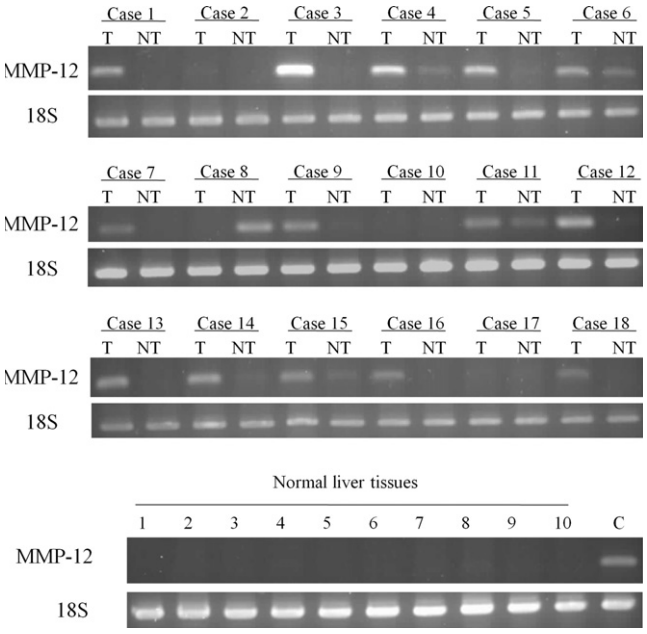

Fig. 1 – RT-PCR analysis of MMP-12 transcript among liver tissues of (A) HCC patients and (B) normal donors. T, tumour tissue; NT, non-tumour tissue; C, positive control.

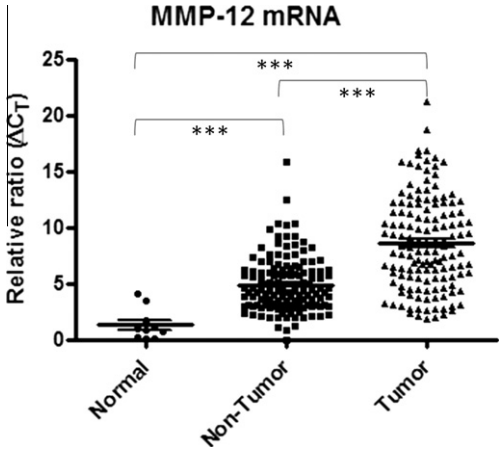

Fig. 2 – Relative expression ratio of MMP-12 mRNA among normal liver tissues and HCC patients by real-time semi-quantitative RT-PCR analysis. ***P < 0.0001.

To define differential expression pattern of MMP-12 mRNA among HCC patients, the difference of MMP-12 mRNA between pair of tumour and non-tumour liver tissues of each HCC patients was determined as ΔΔΔCt(patient). Among 139 HCC patients, 105 patients (75.54%) were found to differentially upregulate MMP-12 mRNA (the value of ΔΔΔCt (patient) $\geqslant 1$) in tumour tissues compared to non-tumour tissues (data not shown).

3.2. *Clinical significance of MMP-12 mRNA in HCC patients*

To obtain a optimal cutoff point to define whether MMP-12 is overexpressed in HCC patients, ROC curve was generated to

analysis the sensitivity and 1-specificity using the value of ΔΔΔCt(patient) to predict 1-year overall survival of HCC patients post-operation. The value of under area of the ROC curve was calculated to be 0.735. According to Youden index, the optimal cutoff value of ΔΔΔCt(patient) among patients was determined to be ΔΔΔCt(patient) = 3.02. Eighty HCC patients (57.55%) whose values of ΔΔΔCt(patient) of MMP-12 mRNA were higher than 3.02 were defined as MMP-12 overexpression group while other 59 HCC patients (42.45%) were defined as MMP-12 non-overexpression group.

The expression pattern of MMP-12 mRNA in HCC patients (overexpression versus non-overexpression) was analysed with their clinicopathological parameters by chi-square test (Table 1). Overexpression of MMP-12 mRNA was significantly correlated with presence of venous infiltration ($p = 0.004$), high serum AFP level ($p = 0.009$) and appearance of recurrence within 1st year ($p = 0.018$). No significant association was obtained between MMP-12 mRNA and sex, age, pTNM stage, cirrhosis, tumour size and hepatitis B surface antigen.

3.3. *Overexpression of MMP-12 mRNA correlated with poor overall survival of HCC patients after hepatectomy*

Kaplan–Meier analysis was applied to examine the prognostic value of MMP-12 mRNA to overall survival and disease-survival of HCC patients after hepatectomy. HCC patients with overexpression of MMP-12 mRNA were significantly associated with poor overall survival (log rank = 5.379, $p = 0.020$, Fig. 3A). The mean of overall survival period of HCC patients after hepatectomy in the overexpression group and the non-overexpression group was 65 and 87 months, respectively. The mean of disease-free survival period of HCC patients after hepatectomy in the overexpression group and the non-overexpression group was 48 and 58 months, respectively.

Table 1 – Correlation of MMP12 mRNA and clinicopathological features of HCC patients.

Clinicopathological features	Number (*n*)	MMP12 mRNA (*n*)		*P*
		Non-overexpression	Overexpression	
Sex				
Male	113	51	62	0.182
Female	26	8	18	
Age				
⩽55 years	78	32	46	0.702
>55 years	61	27	34	
pTNM stage[a]				
Early stage (I–II)	40	21	19	0.111
Advanced stage (III–IV)	98	37	61	
Venous infiltration				
Absent	56	32	24	0.004**
Present	83	27	56	
Cirrhosis[a]				
No	49	20	29	0.788
Yes	88	38	50	
Encapsulation[a]				
Absent	35	11	24	0.142
Present	26	13	13	
Tumour size[a]				
<5 cm	40	17	23	0.955
⩾5 cm	79	34	45	
AFP level[a]				
⩽1000 ng/ml	104	51	53	0.009**
>1000 ng/ml	34	8	26	
Hepatitis B surface antigen[a]				
Negative	18	7	11	0.743
Positive	121	52	69	
Recurrence within 1st year[b]				
No	78	42	36	0.018*
Yes	52	17	35	

PTNM = pathologic tumour-node-metastasis; AFP = alpha-fetoprotein.

* $P < 0.05$.

** $P < 0.01$.

[a] Total number less than 139 due to missing data.

[b] Nine patients were excluded because of their death within the first year without recurrence.

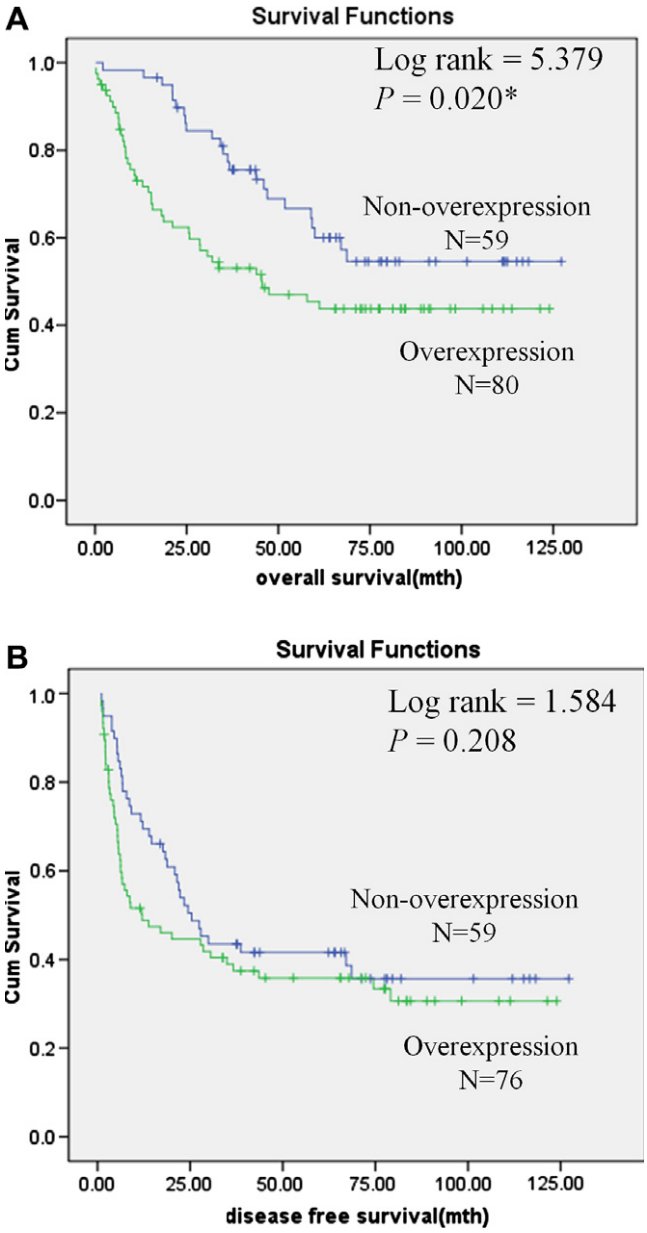

Fig. 3 – Kaplan–Meier analyses of HCC patients. (A) Overall survival and (B) disease free survival of HCC patients in correlation with MMP-12 mRNA expression were analysed by Kaplan–Meier analyses. *Statistical significance.

However, there is no significant correlation between MMP-12 mRNA and disease-free survival of HCC patients after hepatectomy (log rank = 1.584, $p = 0.208$, Fig. 3B).

Cox proportional hazard regression analysis was employed to find out the independent predictors for predicting overall survival of HCC patients after hepatectomy among selected 4 factors including MMP-12 mRNA, pTNM stage, venous infiltration and serum AFP level which were significantly associated with the overall survival of HCC patients by Kaplan–Meier analysis (data not shown). Univariable Cox proportional hazard regression analysis showed that MMP-12 mRNA (HR = 1.81, 95% CI = 1.09–3.02, $p = 0.022$) and other factors were significantly associated with overall survival of HCC patients after hepatectomy (Table 2). Multivariable Cox proportional hazard regression analysis revealed that there is no independent factor among factors to predict overall survival of HCC patients (Table 2). Logistic regression analysis of these factors for predicting 1-, 3- and 5-year overall survival of HCC patients showed that MMP-12 mRNA was significant predictor for predicting 1-year (OR = 20.58, 95% CI = 2.61–162.24, $p = 0.004$) and 3-year (OR = 2.68, 95% CI = 1.16–6.20, $p = 0.021$) overall survival of the HCC patients (Table 3).

4. Discussion

MMP-12, a subfamily of macrophage metalloelastase of the family of matrix metalloproteinase, exhibits the ability to degrade elastin, an important structural protein for many tissues such as blood and lung tissues. The role of MMP-12 on cancer, favourable or unfavourable for tumour growth and metastasis, depends on its context.[8–12] From our previous animal study, MMP-12 gene has been found to be overexpressed in more progressive and invasive rat tumour.[5] Deregulation of MMP-12 gene has been recognised in different human cancers.[18–25] In our study, the expression level of MMP-12 mRNA in normal donors was very low. However MMP-12 mRNA expression levels were increased by 5-fold in non-tumour liver tissues and further elevated to more than 8-fold in tumour liver tissues of HCC patients (Fig. 2). The data indicated their possible participation on cancer development from dysplastic nodules to neoplastic lesions. Together with the above evidence, it was thus proposed that MMP-12 may play important roles in HCC development and metastasis.

The associations between MMP-12 overexpression in tumours and clinicopathological parameters of patients are diverse in different cancers. In contrast to Gorrin-Rivas et al.[32] in which no statistical significance was found between MMP-12 mRNA overexpression in tumour and any clinical variables of Japanese HCC patients, our result demonstrated that overexpression of MMP-12 in tumour tissue was significantly associated with the presence of venous infiltration and high serum AFP level of HCC patients. Venous infiltration and serum AFP level are two important indicators of tumour malignancy of HCC patients. Thus, the result indicated a positive linkage between MMP-12 overexpression in tumour and tumour progression in HCC patients. Although overexpression of MMP-12 mRNA was not significantly associated with advanced stage, 61 out of 98 (62.24%) advanced stage HCC patients were detected to overexpress MMP-12 mRNA in tumour liver tissues (Table 1), suggesting its possible role on promoting tumour progression. Furthermore, our findings demonstrated a statistical significance between MMP-12 overexpression and early tumour recurrence of HCC patients after hepatectomy. In non-small cell lung cancer (NSCLC), MMP-12 overexpression is also significantly correlated with local recurrence and metastasis,[19] suggesting a common role in tumour recurrence in different cancers. High incidence of tumour recurrence continues to be a problem, shortening the lifespan of HCC patients after surgical resection.[4] Thus, the positive linkage between MMP-12 overexpression and early tumour recurrence may not only be used for identifying HCC patients with higher risk of early tumour recurrence

Table 2 – Cox proportional hazard regression analysis of MMP12 mRNA expression and clinicopathological parameters in relation to the overall survival of HCC patients.

	Univariable analysis		Multivariable analysis	
	HR (95% CI)	P	HR (95% CI)	P
MMP12 mRNA				
Overexpression versus non overexpression	1.81(1.09–3.02)	0.022*	1.30(0.76–2.23)	0.334
pTNM stage				
Advanced versus early	4.98(2.36–10.54)	0.000*	2.71(0.93–7.90)	0.068
Venous infiltration				
Presence versus absence	4.63(2.49–8.58)	0.000*	2.37(0.98–5.69)	0.054
AFP level				
>1000 ng/ml versus ⩽1000 ng/ml	1.763(1.04–3.00)	0.037*	1.36(0.79–2.35)	0.264

HR = hazard ratio; CI = confidence interval.
* Statistical significance.

Table 3 – Logistic regression analysis of MMP12 mRNA and clinicopathological parameters on predicting the 1-, 3- and 5 year overall survival of HCC patients.

	1-Year survival		3-Year survival		5-Year survival	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
MMP12 mRNA						
Overexpression versus non-overexpression	20.58(2.61–162.24)	0.004*	2.68(1.16–6.20)	0.021*	0.93(0.37–2.33)	0.875
pTNM stage						
Advanced stage versus early stage	4.17(0.30–57.46)	0.286	7.17(1.52–33.88)	0.013*	6.41(1.81–22.70)	0.004*
Venous infiltration						
Presence versus absence	3.20(0.44–23.15)	0.250	2.03(0.64–6.39)	0.227	4.38(1.40–13.69)	0.011*
AFP level						
>1000 ng/ml versus ⩽1000 ng/ml	0.10(0.35–2.84)	0.996	1.55(0.63–3.84)	0.342	0.93(0.34–2.57)	0.886

OR = odd ratio; CI = confidence interval.
* Statistical significance.

but also for providing important clues to understand the possible mechanism of HCC tumour recurrence after surgical operation.

MMP-12 has been considered to be a valuable prognostic indicator for predicting the survival rate of cancer patients who have undergone surgical resection. Patients carrying pancreatic tumours with overexpressed MMP-12 are significantly associated with poorer overall survival after pancreatic resection compared with patients without overexpression of MMP-12 in tumours.[25] Overexpression of MMP-12 mRNA is an independent factor for tumour relapse-free survival of NSCLC patients after lung resection.[19] In our study, overexpression of MMP-12 mRNA was significantly associated with poor overall survival of HCC patients after hepatectomy. Although multivariable Cox proportional hazard regression analysis showed that MMP-12 mRNA was not an independent factor for overall survival, logistic regression analysis showed that MMP-12 mRNA was a significant factor for 1- and 3-year overall survival, indicating that MMP-12 mRNA might be a predictor for short-term overall survival of HCC patients who have undergone surgical resection. However, the study conducted by Gorrin-Rivas's group in Japan has found that overexpression of MMP-12 mRNA is significantly associated with good overall survival of HCC patients after hepatectomy.[32] There might be several reasons contributing to this difference. First, there are geographical variations contributing to different risk factors of hepatocarcinogenesis between these two regions. Moreover, different methods were employed to examine the expression level of MMP-12 mRNA and different criteria were employed to define the "overexpression" of MMP-12 mRNA. Furthermore, patients were recruited at different periods of time that Gorrin-Rivas's study recruited HCC patients from year 1992 to 1997 while our samples were from year 1999 to 2007. This difference may cause changes in tumourigenic factors of HCC patients.

In summary, our data demonstrated that MMP-12 mRNA was commonly upregulated in HCC patients. Overexpression of MMP-12 mRNA in HCC tumour was significantly associated with venous infiltration of tumour, high level of serum AFP level, early tumour recurrence and poor overall survival of HCC patients after hepatectomy. Therefore, MMP-12 mRNA could be used as a prognostic marker for predicting the short-term overall survival of HCC patients after liver resection. Moreover, it is certain that the findings from our animal study could be beneficial for clinical application.

Conflict of interest statement

None declared.

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