# Matrix metalloproteinase 12 expression is associated with tumor FOXP3+ regulatory T cell infiltration and poor prognosis in hepatocellular carcinoma

MIN-KE HE[1,2*], YONG LE[1,2*], YONG-FA ZHANG[1,2], HAN-YUE OUYANG[1-3], PEI-EN JIAN[1,2], ZI-SHAN YU[1,2], LI-JUAN WANG[1,2] and MING SHI[1-3]

[1]Department of Hepatobiliary Oncology; [2]Collaborative Innovation Center for Cancer Medicine, Cancer Center, Sun Yat-Sen University; [3]State Key Laboratory of Oncology in South China, Guangzhou, Guangdong 510060, P.R. China

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**Abstract.** Hepatocellular carcinoma (HCC) is among the most fatal types of cancer worldwide due to its high rates of recurrence and metastasis. The molecular processes involved in HCC progression require further investigation to identify biomarkers for use in diagnosis and treatment. In the present study, the significance and prognostic value of matrix metallopeptidase 12 (MMP12) expression in human HCC was investigated. MMP12 mRNA expression was investigated using reverse transcription-quantitative polymerase chain reaction analysis of 42 pairs of tumor and non-tumor liver tissues obtained from patients with HCC following surgical treatment. Immunohistochemical staining was used to detect MMP12 and forkhead box P3 (FOXP3) expression in 158 paraffin-embedded HCC tissues. The prognostic value of MMP12 expression was determined using Kaplan-Meier analysis and the Cox proportional hazards model. MMP12 mRNA levels were significantly higher in liver tumor tissues compared with matched non-tumor liver tissues. MMP12 expression and FOXP3+ regulatory T cell (Treg) infiltration was positively correlated (r=0.302; P<0.001). MMP12 protein overexpression was positively correlated with tumor size (P=0.018), high serum alpha-fetoprotein levels (P=0.005) and poor overall survival time (P=0.012) in patients with HCC. Furthermore, MMP12 protein level was an independent predictive factor for overall survival time of patients with HCC who underwent curative resection. In conclusion, these results suggest that MMP12 may increase FOXP3+ Treg infiltration into tumor tissues, and promote tumor progression and immune evasion of HCC. The overexpression of MMP12 protein is, therefore, a valuable prognostic indicator in patients with HCC.

*Correspondence to:* Dr Ming Shi, Department of Hepatobiliary Oncology, Cancer Center, Sun Yat-Sen University, 651 Dongfeng Donglu, Guangzhou, Guangdong 510060, P.R. China
E-mail: shiming@sysu.edu.cn

*Contributed equally

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## Introduction

Hepatocellular carcinoma (HCC) is among the most prevalent types of malignant neoplasm, and its incidence and mortality rates have progressively increased worldwide (1,2). There are >700,000 new cases diagnosed globally each year (1). The majority of HCC cases are associated with established risk factors, including chronic viral hepatitis and alcohol abuse (3). Hepatitis infection is common in China, where HCC is also very commonly diagnosed and among the leading causes of cancer-associated mortality (4). Despite improvements in the diagnosis and treatment of HCC, the overall survival (OS) time of patients with HCC remains poor (5,6). Therefore, the discovery of novel tumor biomarkers is required to aid the devising of optimal treatment strategies and to improve the prognosis of patients with HCC.

Human matrix metallopeptidase 12 (MMP12), also known as macrophage metalloelastase, was first identified in human alveolar macrophages (7). MMP12 belongs to a family of zinc-dependent proteases that are involved in the degradation of extracellular matrix components (8). The inactive form of human MMP12 is a 54-kDa protein, which is processed to generate a 45-kDa form and, subsequently, a 22-kDa active form via loss of its N- and C-terminal residues (7). MMP12 serves crucial roles in various diseases, including chronic obstructive pulmonary disease (9), skin diseases (10), aneurysms (11) and cancer (12). However, the mechanism of MMP12 activity remains controversial in various types of cancer. Antitumor effects of MMP12 have been demonstrated in gastric (13) and colorectal cancer (14). In contrast, MMP12 has been demonstrated to be overexpressed, and associated with tumor occurrence and progression, in non-small cell lung cancer (15), skin cancer (10), ovarian cancer (16), esophageal squamous cell carcinoma (12) and pancreatic cancer (17).

Regulatory T cells (Tregs) were first described as suppressor T cells 1970, and demonstrated to serve important roles in maintaining immune tolerance and controlling

inflammatory diseases (18,19). Forkhead box P3 (FOXP3) functions as a master regulator during the development and control of Tregs (20,21). It is widely viewed as the most specific and reliable surface marker of Tregs (22-24). FOXP3-expressing Tregs, which suppress aberrant immune responses against self-antigens, also suppress the antitumor immune response (25). In humans, the infiltration of large numbers of Tregs into tumor tissues is considered a biomarker and prognostic indicator of malignant tumors (26).

In the present study, the expression of MMP12 and infiltration of FOXP3-expressing ($FOXP3^+$) Tregs were investigated in whole-tissue sections obtained from a cohort of 158 patients with HCC. Furthermore, the prognostic significance of MMP12 protein expression in HCC was investigated.

## Materials and methods

*Patients and samples*. The present study was approved by the Research Ethics Committee of Sun Yat-Sen University Cancer Center (Guangzhou, China). All participants provided written informed consent according to the Declaration of Helsinki (27). Paired tumor and non-tumor tissues were obtained from 42 patients who underwent HCC resection at Sun Yat-Sen University Cancer Center (Guangzhou, China) between January 2015 and December 2016. The HCC tissue microarrays consisted of tissues obtained from 158 patients who were diagnosed with HCC between October 2005 and December 2011 at Sun Yat-Sen University Cancer Center (Guangzhou, China). The tissue microarray was constructed according to a previously described method (28). The inclusion criteria for patient enrollment were as follows: i) Histologically confirmed diagnosis; ii) no distant metastasis; iii) no neoadjuvant chemotherapy or radiotherapy prior to surgery; iv) no serious complications or other malignant diseases, and v) complete follow-up data available. The tumor stage was determined according to the 7th Edition Tumor-Node-Metastasis (TNM) classification system (29). Tumor differentiation was graded according to the Edmondson grading system (30). The clinicopathological characteristics of the patients are summarized in Table I.

*Immunohistochemistry (IHC)*. IHC was performed as described in our previous study (26). Briefly, the sections were deparaffinized in dimethyl benzene, then progressively rehydrated in 100, 95, 90, 80 and 70% ethanol solutions. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% hydrogen peroxide at room temperature for 10 min. Subsequently, the slides were boiled (100˚C, 5 min; 95˚C, 25 min) in citrate-hydrochloric acid (pH 6.0) for heat-induced epitope retrieval. The tissue sections were then incubated with an MMP12 primary antibody (dilution, 1:200 cat. no. ab52879) and a FOXP3 primary antibody (dilution, 1:100; cat. no. ab20034; both Abcam, Cambridge, UK) overnight at 4˚C. A horseradish peroxidase-conjugated anti-rabbit/mouse Dako REAL™ EnVision™ detection system (cat. no. K5007; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) was then used according to the manufacturer's protocol. Finally, the sections were counterstained with hematoxylin at room temperature for 2 min. A negative control was provided by replacing the primary antibody with normal rabbit IgG (cat. no. 12-370; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). To assess the expression level of MMP12/FOXP3, a Vectra-inForm image analysis system (PerkinElmer, Inc., Waltham, MA, USA) was used as described in previous studies (31,32).

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. Total RNA was isolated from tumor and non-tumor liver tissues using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. RNA concentrations were calculated using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Inc.). RNA with an absorbance ratio from 1.8-2.0 at 260 and 280 nm (260/280) was regarded as pure. cDNA was synthesized from 2 $\mu$g pure total RNA using a Revert Aid First-Strand cDNA Synthesis kit (Toyobo Life Science, Osaka, Japan). The resulting cDNA was then subjected to RT-qPCR to determine relative MMP12 mRNA expression levels. The reference gene, GAPDH, served as an internal control. RT-qPCR assays (ReverTra Ace® qPCR RT Master Mix; cata. no. FSQ-201; Toyobo Life Science) were performed in triplicate at a final volume of 10 $\mu$l. The reactions consisted of 5 $\mu$l 2X SYBR Green master mix (Toyobo Life Science), 0.4 $\mu$l 20 mmol/l forward primer, 0.4 $\mu$l reverse primer, 0.75 $\mu$l sample cDNA and 3.45 $\mu$l RNase-free water. The thermocycling conditions used were as follows: An initial step in which the mixture was preheated to 95˚C for 10 min, followed by 45 cycles at 95˚C for 30 sec and 60˚C for 60 sec. The following specific primers were used: MMP12 forward, 5'-CCCTGG TTATCCCAAACTGA-3' and reverse, 5'-CCAAACCAGCTA TTGCTTTTC-3'; and GAPDH forward, 5'-CTCCTCCTGTTC GACAGTCAGC-3' and reverse, 5'-CCCAATACGACCAAA TCCGTT-3' (Beijing Ruibiotech Co., Ltd., Beijing, China). The data were analyzed using the comparative threshold cycle ($2^{-\Delta\Delta Cq}$) method (33) and Roche LightCycler 480 software (version 1.5; Roche Diagnostics, Basel, Switzerland), and the results were averaged, normalized and expressed in relative expression units.

*Statistical analysis*. SPSS 20.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism V6.0 (GraphPad Software, Inc., La Jolla, CA, USA) were used for statistical analysis. The differences in MMP12 mRNA levels between HCC tissues and matched non-tumor liver tissues were analyzed using paired t-tests. The association between MMP12 expression status and clinicopathological features was analyzed using $\chi^2$ or Fisher's exact tests, as appropriate. Briefly, if $n<5$, Fisher's exact tests were used; otherwise, $\chi^2$ tests were used. Pearson's correlation coefficient was used to assess the correlation between MMP12 and FOXP3 expression, as analyzed by IHC. OS curves were generated using the Kaplan-Meier method and analyzed using the log-rank test. Parameters demonstrated to be significant in univariate analysis were further assessed in a multivariate Cox proportional hazards model. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*MMP12 expression in hepatocellular carcinoma tissues*. To compare the expression of MMP12 in paired HCC tumor and

Table I. Association between MMP12 expression and clinicopathological characteristics of patients with hepatocellular carcinoma (n=158).

| | | MMP12 expression | | |
|---|---|---|---|---|
| Clinicopathological variable | n | Negative, n | Positive, n | P-value |
| Age, years | | | | |
| ≤50 | 78 | 38 | 40 | 0.429 |
| >50 | 80 | 44 | 36 | |
| Sex | | | | |
| Male | 143 | 75 | 68 | 0.670 |
| Female | 15 | 7 | 8 | |
| Hepatitis B surface antigen | | | | |
| Negative | 18 | 8 | 10 | 0.501 |
| Positive | 140 | 74 | 66 | |
| Serum α-fetoprotein, ng/ml | | | | |
| ≤400 | 93 | 57 | 36 | 0.005 |
| >400 | 65 | 25 | 40 | |
| Liver cirrhosis | | | | |
| No | 100 | 51 | 49 | 0.143 |
| Yes | 58 | 33 | 22 | |
| Tumor size, cm | | | | |
| ≤5 | 63 | 40 | 23 | 0.018 |
| >5 | 95 | 42 | 53 | |
| Tumor number | | | | |
| Solitary | 115 | 59 | 56 | 0.807 |
| Multiple | 43 | 23 | 20 | |
| Microvascular invasion | | | | |
| No | 140 | 75 | 65 | 0.241 |
| Yes | 18 | 7 | 11 | |
| Differentiation grade | | | | |
| I+II | 82 | 42 | 40 | 0.325 |
| III+IV | 76 | 42 | 34 | |
| TNM stage | | | | |
| I | 101 | 54 | 47 | 0.600 |
| II+III | 57 | 28 | 29 | |

MMP12, matrix metallopeptidase 12; TNM, Tumor-Node-Metastasis.

adjacent non-tumor tissues, the MMP12 mRNA expression level was analyzed in 42 pairs of tissue using RT-qPCR. It was demonstrated that the expression level of MMP12 mRNA was significantly higher in HCC tumor tissues compared with the matched adjacent non-tumor tissues (Fig. 1A). IHC staining of 16 specimens was performed, revealing that MMP12 was primarily localized in tumor cell cytoplasm. 1C). The adjacent non-tumor cells were negative for MMP12 expression (Fig. 1B). MMP12 protein was localized in the tumor nuclei of some specimens (Fig. 1). Furthermore, in HCC tissue specimens, it was observed that non-tumor cells also expressed MMP12 protein. Based on previous research (7), it was hypothesized that these cells may be tumor associated-macrophages.

*Associations between MMP12 expression and clinicopathological characteristics.* Patients were divided into the following groups based on MMP12 expression in the tumor cell cytoplasm, determined using the receiver operating characteristic (ROC) curve: MMP12-(negative expression in tumor cells) and MMP12+ (positive expression in tumor cells). These groups included 51.9 and 48.1% of all included samples, respectively. Patients in the MMP12+ group exhibited a significantly larger average tumor size (P=0.018) and significantly higher serum α-fetoprotein (AFP) levels (P=0.005) compared with the MMP12-group (Table I). However, there was no significant difference in MMP12 protein expression status in HCC tissues according to age, sex, tumor number, presence of invasive microvasculature, differentiation grade or TNM stage (Table I).

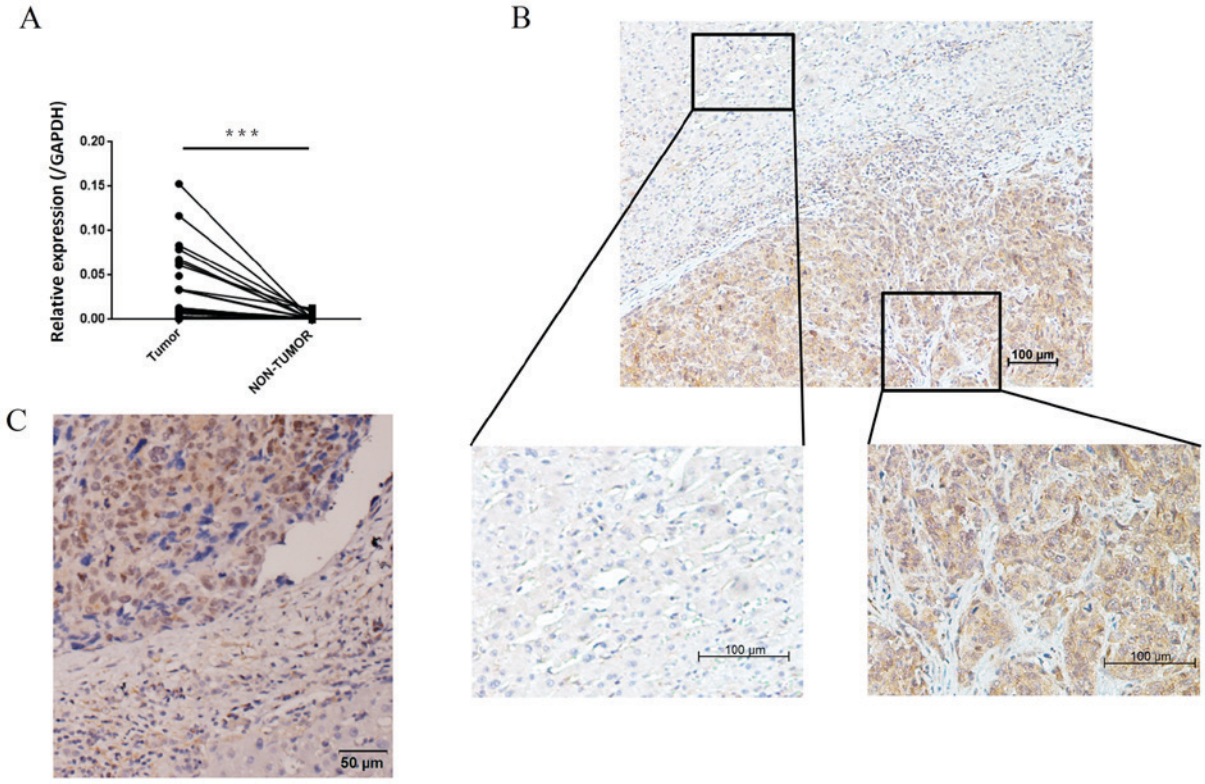


Figure 1. Overexpression of MMP12 in HCC tissue. (A) MMP12 expression was detected by reverse transcription quantitative-polymerase chain reaction in 42 pairs of HCC and non-tumor tissues. The expression of MMP12 was significantly increased in HCC compared with non-tumor tissues. (B) Immunohistochemical analysis of MMP12 in HCC specimens (magnification, x100). Enlarged panels of the indicated areas are also provided (magnification, x200). (C) MMP12 protein was detected in the tumor cell nucleus (magnification, x200). ***P<0.001. MMP12, matrix metallopeptidase 12; HCC, hepatocellular carcinoma.

*Prognostic significance of MMP12 protein expression in HCC patients*. Kaplan-Meier survival analysis revealed that patients in the MMP12+ group had a shorter OS time than patients in the MMP12-group (P=0.012; Fig. 2A). In addition, all patients were stratified according to the TNM staging system. There were 101 patients at stage I, 21 patients at stage II and 36 patients at stage III. In patients with stage I or III tumors, the surgical prognosis more positive in the MMP12-compared with the MMP12+ group (stage I; P=0.018; Fig. 2B, and stage III P=0.049; Fig. 2D). However, this association was not demonstrated in stage II patients (P=0.821; Fig. 2C). To determine whether positive MMP12 expression in tumor cells is an independent prognostic factor for HCC, multivariate survival analysis was performed. As demonstrated in Table II, positive MMP12 expression was an independent prognostic factor for OS in patients with HCC (HR=2.013; 95% CI=1.211-3.347; P=0.007).

*Correlation between MMP12 expression and FOXP3⁺ T cell number in human HCC tissues*. To investigate the association of MMP12 expression and FOXP3 expression in human HCC tissues, IHC staining for MMP12 and FOXP3 was performed in human HCC tissue sections. Tissues positively expressing MMP12 also expressed high levels of FOXP3 (Fig. 3Aa and Ab). Conversely, tissues that did not express MMP12 also expressed low levels of FOXP3 (Fig. 3Ac and Ad). Statistical analysis indicated a significantly positive correlation between MMP12 expression and FOXP3 expression (r=0.302, P<0.001; Fig. 3B).

## Discussion

In the present study, it was demonstrated that MMP12 protein was usually in tumor cell cytoplasm, but was sometimes localized in the nucleus. According to RT-qPCR analysis, MMP12 was expressed at significantly higher levels in HCC tissues than in non-tumor tissues, and MMP12 overexpression was also associated with malignant clinicopathological characteristics, including larger tumor size and high serum AFP levels. However, there was no association between MMP12 expression in HCC tissues and patient age, sex, tumor number, microvascular invasion or TNM stage. In the present study, MMP12 expression was positively correlated with tumor size but not with tumor number or microvascular invasion. Therefore, MMP12 expression was not significantly correlated with TNM stage. These results are in agreement with a previous study (34). It was also demonstrated that this pattern of MMP12 protein expression in tumor cells was associated with a relatively poor prognosis. Furthermore, MMP12 protein expression level and FOXP3⁺ Treg infiltration were positively correlated. Taken together, these conclusions suggest that MMP12 may not serve important roles in metastasis, but may affect prognosis by influencing the immune system in HCC patients.

In a previous study, the overexpression of MMP12 mRNA was demonstrated to be associated with the presence of venous infiltration, high serum AFP levels, early tumor recurrence and poor overall survival time in patients with HCC (34). However, the results of a different study indicated that MMP12 mRNA

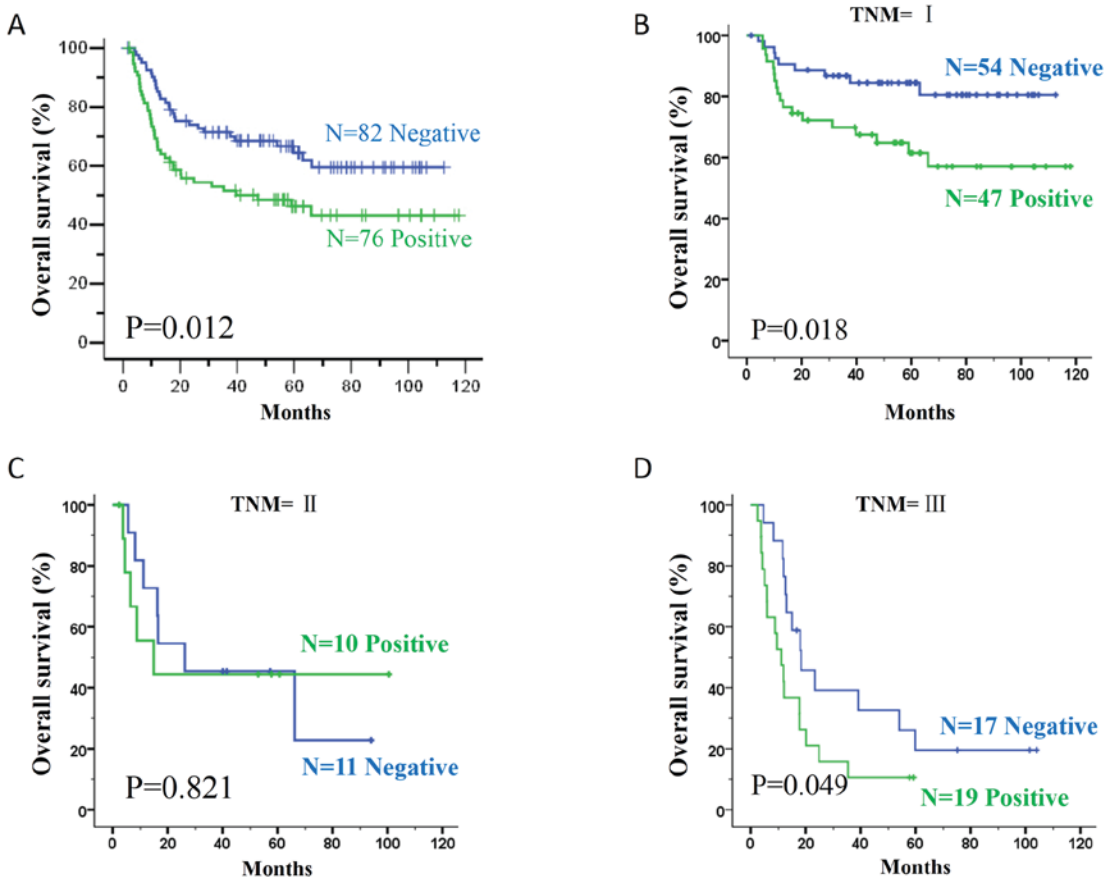


Figure 2. Prognostic significance of MMP12 protein expression in patients with HCC. (A) Kaplan-Meier analysis of the association of MMP12 expression and overall survival time. Analysis of MMP12 expression in tumor cells on the prognoses of patients with TNM stage (B) I, (C) II and (D) III HCC. MMP12, matrix metallopeptidase 12; HCC, hepatocellular carcinoma; TNM, Tumor-Node-Metastasis.

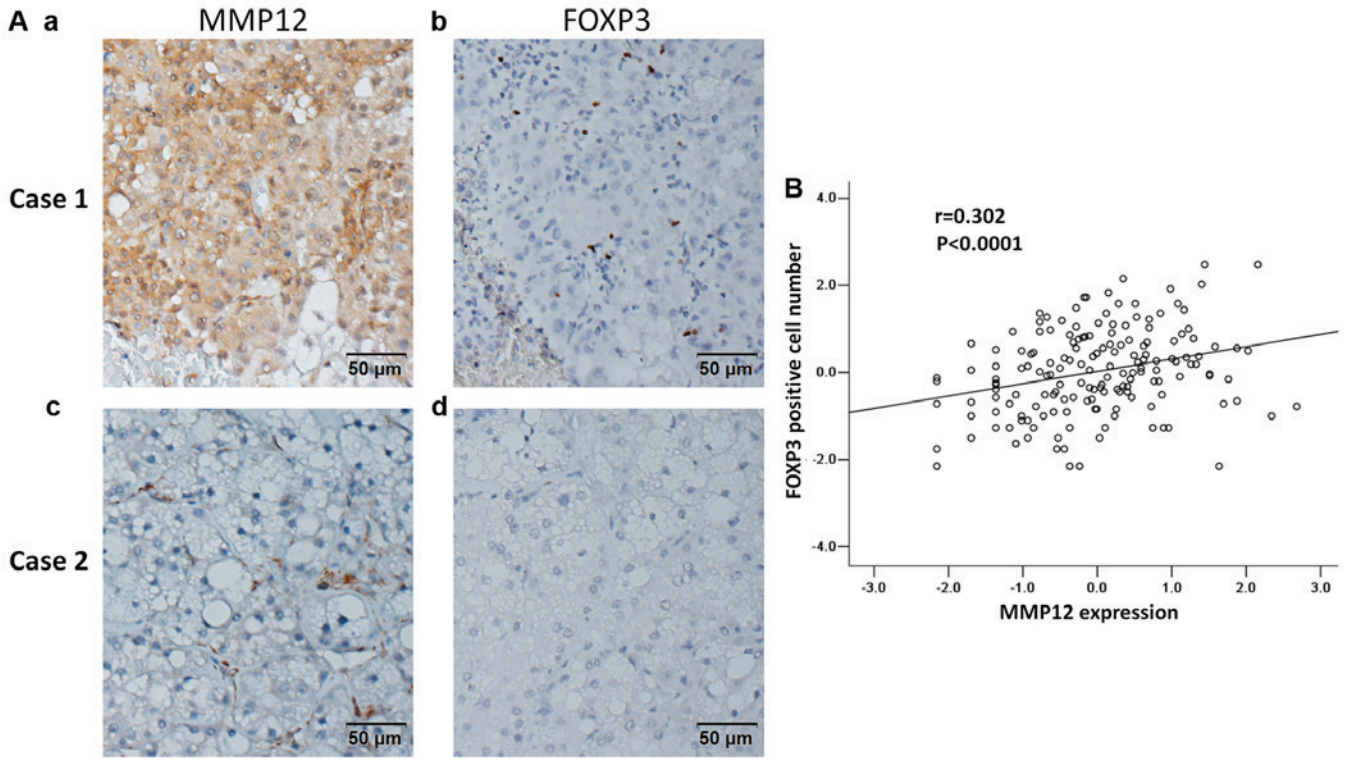


Figure 3. Correlation between MMP12 expression and FOXP3$^+$ Treg infiltration in HCC tissues from 2 patients. (A) Representative images of immunohistochemistry staining of HCC tissue sections for (a/c) MMP12 and (b/c) FOXP3 (x200, magnification). (B) Pearson's correlation coefficient analysis of the association between MMP12 and FOXP3 expression in HCC tissues. MMP12, matrix metallopeptidase 12; FOXP3, forkhead box 3; Treg, regulatory T cells.

Table II. Univariate and multivariate analyses of prognostic factors in hepatocellular carcinoma (n=158).

| | Overall survival | | |
|---|---|---|---|
| Variable | Univariate analysis P-value | Multivariate analysis hazard ratio (95% CI) | P-value |
| Age, years | | | |
| ≤50 | 0.284 | | |
| >50 | | | |
| Sex | | | |
| Male | 0.955 | | |
| Female | | | |
| Serum α-fetoprotein, ng/ml | | | |
| ≤400 | 0.192 | | |
| >400 | | | |
| Liver cirrhosis | | | |
| Negative | 0.001 | 1 | |
| Positive | | 1.907 (1.154-3.153) | 0.012 |
| Tumor size, cm | | | |
| ≤5 | <0.001 | 1 | |
| >5 | | 2.224 (1.234-4.009) | 0.008 |
| Tumor number | | | |
| Solitary | <0.001 | 1 | <0.001 |
| Multiple | | 4.844 (2.927-8.015) | |
| Microvascular invasion | | | |
| No | <0.001 | 1 | 0.001 |
| Yes | | 2.961 (1.545-5.675) | |
| Differentiation grade | | | |
| I+II | 0.139 | | |
| III+IV | | | |
| MMP12 expression | | | |
| Negative | 0.012 | 1 | 0.007 |
| Positive | | 2.013 (1.211-3.347) | |

MMP12, matrix metallopeptidase 12; CI, confidence interval.

overexpression was significantly associated with an improved overall survival time in patients with HCC who underwent curative resection (35). This study also demonstrated that the expression of MMP12 was positively correlated with angiostatin production and hypovascular tumors. However, while these studies determined MMP12 expression levels using a quantitative method, neither detected MMP12 protein expression levels in tumor tissues. The results of the present study suggest that the MMP12 protein is usually localized in the cytoplasm of tumor cells. Furthermore, multivariate analysis revealed that MMP12 expression was an independent and significant risk factor affecting overall survival time of patients with HCC who underwent curative resection. Matrix metallopeptidases (MMPs) are secreted proteases that degrade the extracellular matrix during various cellular processes. Several studies have demonstrated that MMPs are located in the nucleus of human cells (36,37). In one study, MMP12 was demonstrated to be trafficked into the nucleus, where it bound specific DNA sequences to regulate the expression of specific genes (37). In the present study, it was observed that the MMP12 protein was localized in tumor cell nuclei in HCC specimens. The transcriptional functions of MMP12 in HCC should be investigated, in order to have confidence that the intracellular localization of MMP12 is not an artifact.

MMPs can activate, deactivate or modify the activities of signaling cytokines, chemokines and receptors acting as modulators of inflammation and innate immunity (38,39). In a bi-transgenic mouse model, MMP12 overexpression in the myeloid lineage cells contributed to modulation of myelopoiesis, immune suppression and lung tumorigenesis (40). In the same study, the number of Tregs was increased in bi-transgenic mice overexpressing MMP12. In the present study, the association between MMP12 expression and FOXP3 expression (FOXP3 is a marker of Tregs) was analyzed in HCC tissues. A positive correlation between MMP12 expression and FOXP3 expression was identified. The upregulated expression level of MMP12 in HCC patients indicated they were in a state of immune suppression.

In summary, the results of the present study suggest that MMP12 mRNA and protein expression levels are higher in HCC patients. In HCC tumor tissues, MMP12 protein overexpression was associated with poor overall survival time in patients with HCC who had undergone hepatectomy. However, the precise mechanism underlying the effect of MMP12 expression on the occurrence and progression of HCC remains to be investigated.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

All authors have read and approved the manuscript. MKH designed the study, performed the experiment and statistical analyses, and wrote the manuscript. YL participated in the design of the study, performed part of the statistical analyses and helped draft the manuscript. YFZ, HYO, PEJ, ZSY and LJW participated in clinical data collection. MS supervised the whole study, was involved in the study design and supervised the draft of the manuscript.

**Ethics approval and consent to participate**

The present study was approved by the Research Ethics Committee of Sun Yat-Sen University Cancer Center. All participants provided written informed consent according to the Declaration of Helsinki.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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