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Matrix metalloproteinase 12 (MMP12) as an adverse prognostic biomarker of vascular invasion in hepatic cell carcinoma

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Abstract. – OBJECTIVE: Vascular invasion is closely associated with tumor recurrence and poor outcomes in hepatocellular carcinoma (HCC). In this study, we evaluated the potential prognostic value of matrix metalloproteinase-12 (MMP12) as a biomarker of vascular invasion in HCC patients.

MATERIALS AND METHODS: The Gene Expression Omnibus GSE77509 and TCGA Liver Hepatocellular Carcinoma datasets were analyzed to explore the relationships between genes, vascular invasion, and patient survival. The role of MMP12 in HCC was analyzed in terms of DNA methylation, immune cell infiltration, and patient survival, as well as *in silico* analysis.

RESULTS: Overexpression of MMP12 was associated with poor prognosis in HCC patients with vascular invasion. MMP12 was identified as an independent predictor of overall survival (OS) (HR 2.543; 95% CI 1.224, 5.285; $p = 0.012$) and disease-free survival (DFS) (HR 2.034; 95% CI 1.160, 3.566; $p = 0.013$) in multivariate Cox analysis in HCC patients. MMP12 expression, vascular invasion, tumor status, and AJCC T stage were independent predictors of OS with a concordance index (C-index) of 0.713 (95% CI, 0.671, 0.756). MMP12 expression was related to hypomethylation status and positively correlated with tumor immune cell infiltration and the expression of immune cell-related biomarkers.

CONCLUSIONS: Upregulation of MMP12 was associated with poor prognosis and vascular invasion in HCC. These data suggest that MMP12 may have potential as a therapeutic target and biomarker in HCC.

Key Words:
Biomarkers, Hepatocellular carcinoma, MMP12, Prognosis, Vascular invasion.

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related mortality in East Asia and the sixth leading cause of cancer deaths in Western nations[1,2]. Patients with HCC have high rates of postoperative tumor recurrence with a 77-100% 5-year cumulative recurrence rate. Between 80% and 95% of patients have recurrence in the liver with an associated 5-year survival rate of < 15%[3]. Vascular invasion is a key predictor of tumor recurrence due to its association with the dissemination of tumor cells and poor patient survival outcomes[4,5]. The mechanisms governing HCC onset, progression, and vascular invasion require further investigation and may inform the development of improved biomarkers to better predict invasion. The development of novel molecular approaches for the diagnosis and prognosis of HCC patients is vital towards improving the survival and quality of life for these patients[6,7].

Matrix Metalloproteinase-12 (MMP12) is a member of the peptidase M10 family of matrix metalloproteinases (MMPs) that are found on chromosome 11q22[8]. Proteins in this family are involved in the breakdown of the extracellular matrix during normal physiological processes and tumor metastasis[9]. Heterogeneous nuclear ribonucleoprotein K can induce the expression of MMP12 and promote migration and invasion in nasopharyngeal carcinoma[10]. In gastric cancer, MMP12 upregulation is associated with poorer overall survival (OS) outcomes[11] and more advanced diseases in the context of TNM staging and TGF-β1 expression[12]. The P38 mitogen-activated protein kinase-dependent NF-κB pathway is activated in gastric cancer cells and is also associated with MMP12-related stimulation[13]. Other preliminary data suggest that MMP12 may also promote the growth of lung cancer[14]. While high MMP12 expression has been linked to poor prognosis in liver cancer patients[15], the precise role of MMP12 in HCC vascular invasion remains to be fully understood.

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Genes potentially associated with HCC vascular invasion were investigated in the Gene Expression Omnibus (GEO) dataset GSE77509 and the TCGA liver hepatocellular carcinoma (LIHC) dataset. Differentially expressed genes (DEGs) between normal samples and those from vascular invasion positive (VR+) and vascular invasion negative (VR-) HCC patients were identified. It was found that MMP12 was significantly overexpressed in the VR+ group compared with VR- and normal groups in both databases. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were used to analyze potential functions and investigation of immune infiltration and DNA methylation were performed to determine regulatory mechanisms. The results suggested that upregulation of MMP12 was associated with both poor prognosis and immune infiltration in vascular invasive HCC. MMP12 may thus be a useful biomarker or therapeutic target in HCC.

Materials and Methods

Data Processing

The study process is shown in Figure 1. The GSE77509 dataset included 20 HCC tumors, 20 portal vein tumor thrombosis samples, and 20 paracancerous normal tissue samples (platform, GEO: GPL16791). The data were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/) and used to identify genes associated with vascular invasion. Gene expression patterns and clinical data of HCC patients were downloaded from the TCGA database (https://portal.gdc.cancer.gov/). After normalization of the raw RNA-seq data, target genes were identified from differentially expressed mRNAs (DEmRNAs) using R. The mutation status of MMP12 was evaluated using the cBioPortal for Cancer Genomics (http://www.cbioportal.org/). The study protocol was approved by the Ethics Committees of the First Affiliated Hospital of Jinzhou Medical University.

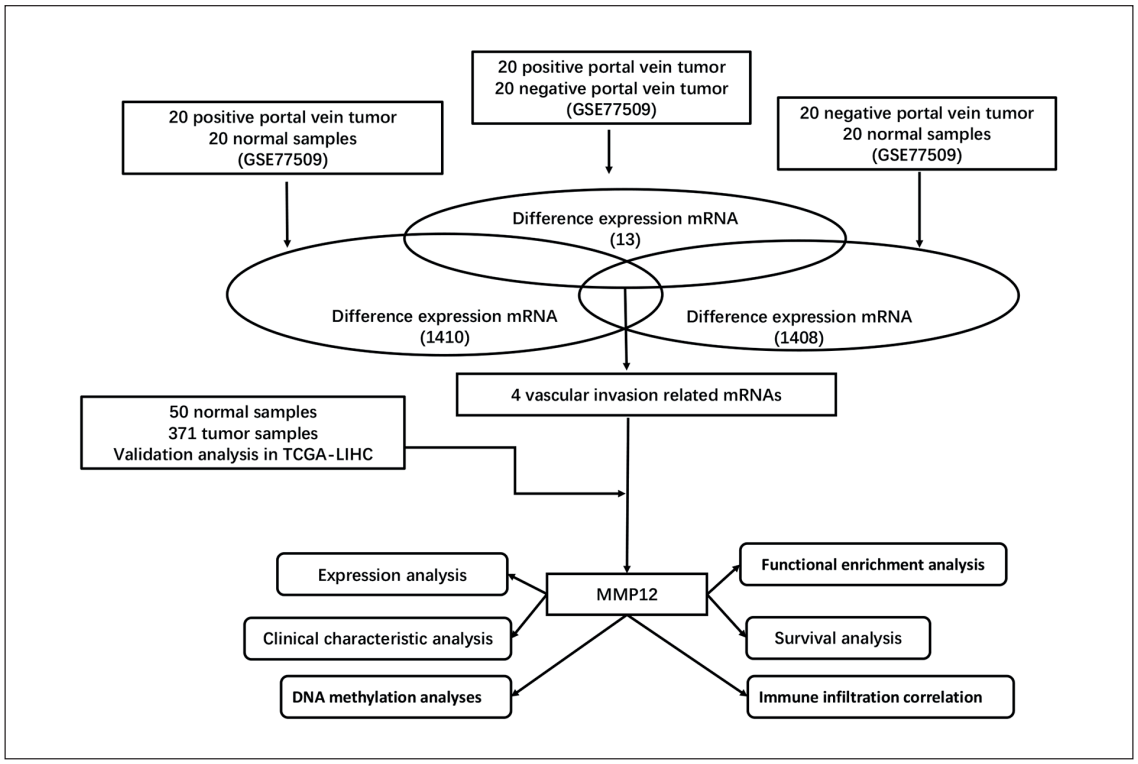

Figure 1. An overview of the study workflow.

Identification of Differentially Expressed mRNAs

Differentially expressed mRNA (DEmRNAs) were identified by comparing samples with and without portal vein invasion. A detection threshold of $|logFC| > 2$ and $p < 0.05$. R (v 3.6) was used to generate volcano plots displaying the differentially expressed mRNAs.

Functional Enrichment Analysis

The identified DEGs were combined with the top 200 MMP12-related genes in the TCGA-LIHC cohort. GO and KEGG enrichment analyses were performed on these genes using Metascape. The data were visualized using the "ggplot2" and "clusterprofile" packages in R. A p-value of < 0.05 was used as the threshold for statistical significance.

Survival Analyses and Development of a Prognostic Model

The survival outcomes were analyzed using the Kaplan-Meier method with a log-rank test for patients with vascular invasion in the TCGA HCC cohort. Univariate Cox regression was conducted to explore the relationships between MMP12 and patient OS to identify prognostic biomarkers. Multivariate Cox regression was then performed to identify factors independently associated with prognosis in HCC patients.

Association Between MMP12 Expression and DNA Methylation Analyses

The expression of MMP12 in HCC tissues and adjacent normal tissues was analyzed using the HCC Database (HCCDB, http://lifeome.net/database/hccdb)[16]. Differences in the expression of MMP12 in the VI+, VI-, and adjacent normal tissues were analyzed in the Oncomine microarray database (https://www.oncomine.com) and

TCGA Dataset

DNA methylation status is a key epigenetic determinant of gene expression that is regulated by the DNA methyltransferases DNMT1, DNMT3A, and DNMT3B. These three DNA methyltransferases can influence cancer cell behavior[17] and are differentially expressed in patients with high and low levels of MMP12 expression in the TCGA database. The UALCAN (http://ualcan.path.uab.edu/) and DiseaseMeth v 2.0 (http://bio-bigdata.hrbmu.edu.cn/diseasemeth/) databases were used to assess MMP12 expression in HCC tumors and paracancerous tissues. The relationships between MMP12 expression and DNA methylation status were examined through MEXPRESS (https://mexpress.be). Also, MethSurv (https://biit.cs.ut.ee/methsurv/) was used for multivariate survival analyses to explore different CpG islands in the context of DNA methylation.

Levels of Immune Infiltration and Expression Analysis of MMP12

The TIMER (https://cistrome.shinyapps.io/timer/) database was used to analyze intratumoral immune cell infiltration based on gene expression data, examining the associations between MMP12 expression infiltration of different immune cell populations including B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells [DCs]. The relationships between these variables and MMP12 copy numbers and their prognostic relevance were assessed in individuals with HCC. Moreover, correlations between MMP12 expression and biomarkers associated with different tumor-infiltrating immune cell types were assessed.

Statistical Analysis

The clinicopathological variables associated with MMP12 expression were analyzed using Pearson chi-squared tests and the Fisher's exact test as appropriate. Disease-free survival (DFS) was defined as the time between surgery and disease recurrence. Overall survival (OS) was defined as the time from diagnosis to mortality or the most recent follow-up. The survival outcomes were compared via Kaplan-Meier curves using a log-rank test. Hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated for DFS and OS using univariate Cox proportional hazards regression analyses. The variables that were significant in the univariate analyses ($p < 0.05$) were incorporated into multivariate analysis. A two-sided $p < 0.05$ was used as the significance threshold for this study. All analyses were performed using R (v 3.6) and GraphPad Prism 8.3, with a p-value threshold of < 0.05 used to indicate statistical significance.

Results

Identification of DEGs Associated with HCC Vascular Invasion

To identify prognostic biomarkers associated with vascular invasion status, we used datasets from the GEO database to identify differentially

expressed mRNAs (DEmRNAs) in HCC samples by comparing VI+ and VI- samples and adjacent paracancerous tissues. A p-value < 0.05 and a |log2(fold-change [FC])| > 2 (DEmRNAs) were used as the criteria for differential expression. Overall, 4317 DEmRNAs were found between VI+ HCC tumors and normal tissues (1410 genes were upregulated, 2907 genes were downregulated). This approach identified 4219 DEmRNAs between the VI+ HCC tumors to normal tissues (1408 genes were upregulated, 2811 genes were downregulated). In addition, 108 DEmRNAs were found between the HCC VI+ and VI- HCC groups (4 upregulated genes and 104 downregulated genes). Volcano plots showing the distributions of the DEmRNAs are shown in Figure 2A, B, and C. Venn diagrams showing the intersections between overexpressed genes and genes associated with vascular invasion are shown in Figure 2D. The four upregulated DEGs between the VI+ and VI- groups were MMP12 (Matrix Metallopeptidase 12), PLPPR4 (Phospholipid Phosphatase Related 4), ACAN (Aggrecan), and C6ORF223 (Chromosome 6 Open Reading Frame 223), identified by GeneCards (https://www.genecards.org).

Analysis of the Prognostic Relevance of MMP12 Overexpression in the Vascular Invasion of HCC Tumors

The HCC database (HCCDB, http://lifeome.net/database/hccdb/) was searched to assess the functional relevance of MMP12 in HCC. This showed that MMP12 was overexpressed at the mRNA level in HCC tumors compared with normal hepatic tissue samples (Figure 3A). Upregulated MMP12 mRNA levels in the VI+ samples compared with VI- and normal samples were also evident in the Wurmbach Liver dataset from Oncomine (Figure 3B). Given the observed upregulation of MMP12 in tumor tissues, we evaluated the clinical relevance of MMP12 overexpression in HCC. Our data showed that higher levels of MMP12 expression were associated with poorer HCC patient OS (Figure 3C).

To explore the potential mechanism for the upregulation of MMP12, copy number variations

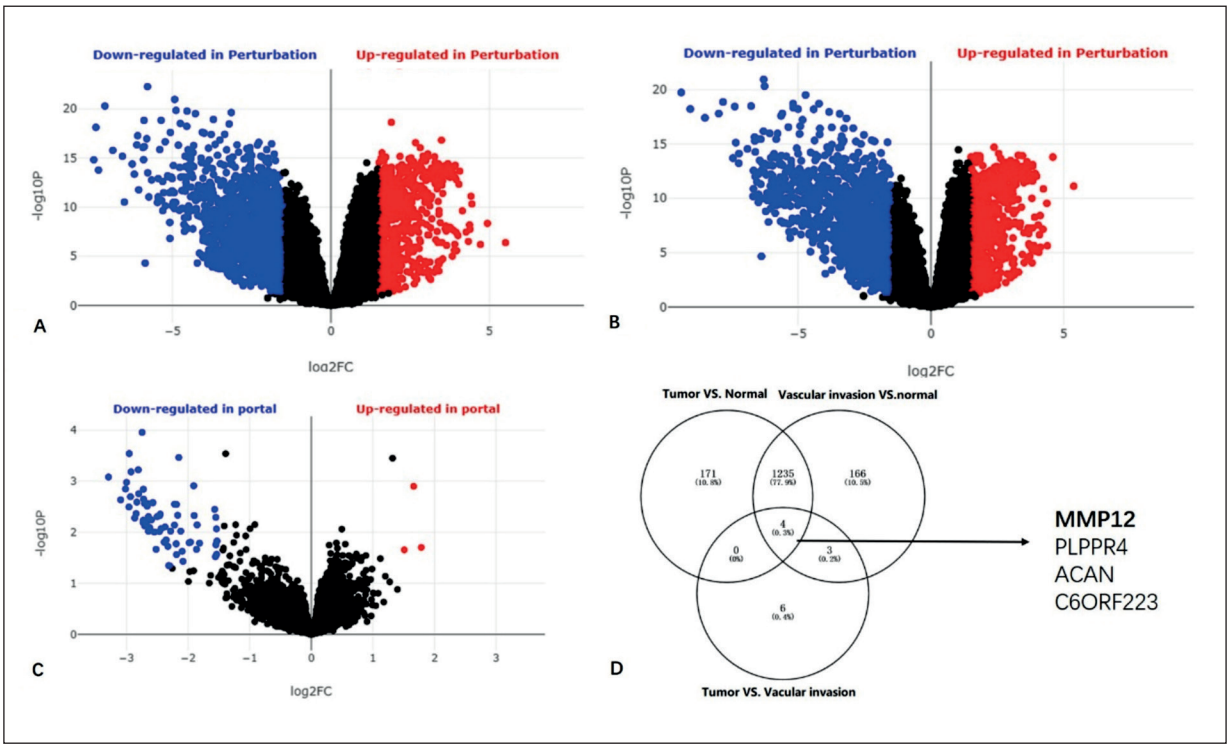

Figure 2. Comparison of differentially expressed mRNAs between HCC tissues with and without vascular invasion (VI+ and VI-) and normal tissues. Red and blue colors correspond to upregulated and downregulated genes, respectively. Volcano plots were generated representing (**A**) differentially expressed mRNAs (|log2(FC) | > 1and adjusted p-value < 0.05) between tumor and normal tissues; **B**, differentially expressed mRNAs between VI+ and normal tissues (|log2(FC)| > 1 and adjusted p-value < 0.05); **C**, differentially expressed mRNAs between VI+ and tumor tissues (|log2(FC)| > 1 and adjusted p-value < 0.05); **D**, Venn diagram of mRNAs related to up-regulated genes involved in tumor vascular invasion of HCC.

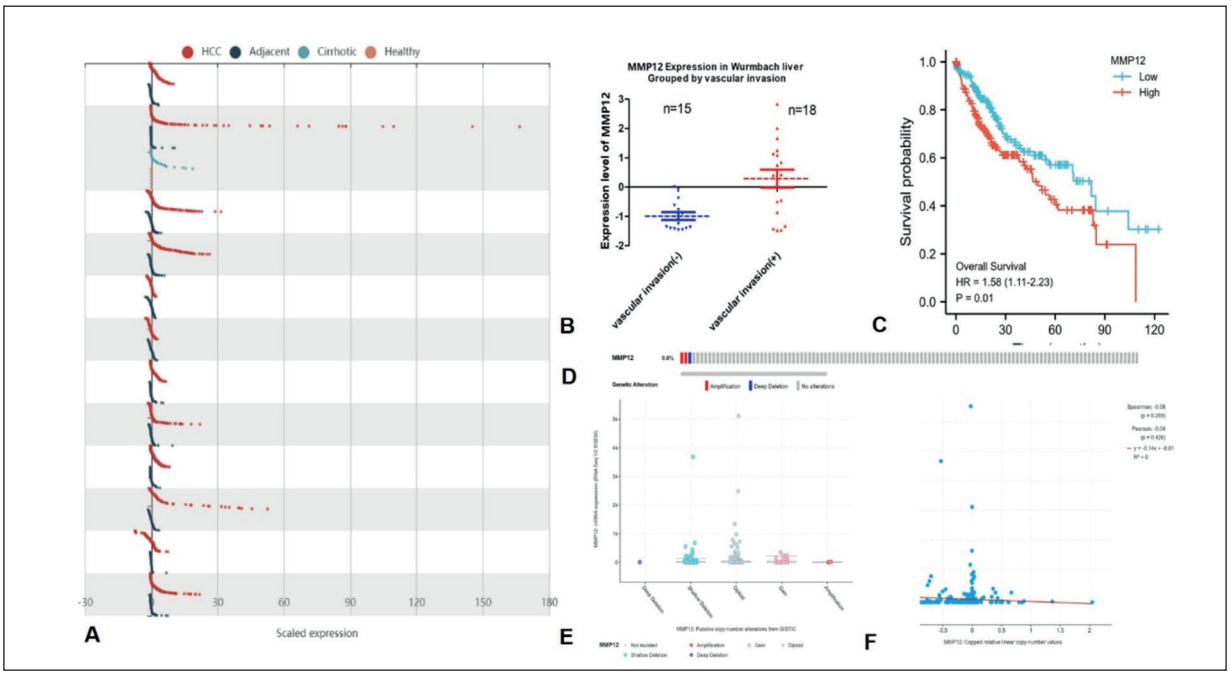

Figure 3. Analysis of MMP12 expression in human HCC. **A**, MMP12 expression in different HCC databases (HCCDB, http://lifeome.net/database/hccdb/). **B**, MMP12 expression in vascular invasion positive/negative and normal samples from the Wurmbach liver dataset in Oncomine; **C**, Overall survival associated with low (n = 185) and high (n = 184) levels of MMP12 expression were compared using Kaplan-Meier curves. **D**, Distribution of MMP12 genomic alterations in the TCGA-HCC cohort shown by an OncoPrint plot from cBioPortal. The relationships between MMP12 copy numbers and mRNA expression levels are represented as dot plots (**E**) and correlation plots (**F**) from cBioPortal.

of MMP12 in HCC were evaluated with cBioPortal (Figure 3D). Approximately 0.8% of HCC samples showed MMP12 amplification. We did not detect any clear correlation between MMP12 copy number and the expression of MMP12 at the mRNA level in HCC patient samples (Figure 3E-F). Together, these data suggest that MMP12 is overexpressed in HCC through a mechanism that is independent of copy number amplification.

To establish if changes in MMP12 expression were associated with patient prognosis, we conducted a series of analyses showing that MMP12 levels were positively correlated with histological grade ($p < 0.001$), T stage ($p = 0.002$), pathological stage ($p = 0.002$), tumor status ($p = 0.013$), and alpha fetoprotein (AFP) level ($p < 0.001$) (Table I).

We then explored the prognostic relevance of these clinical parameters using univariate and multivariate Cox regression to identify predictors of OS and DFS in HCC patients with vascular invasion (n=292). The univariate analysis showed that T stage, tumor status, and MMP12 were associated with both OS ($p < 0.05$; Table II) and DFS ($p < 0.05$; Table III) in the TCGA-HCC cohort. In the multivariate Cox analysis, MMP12 overexpression was independently associated with both lower OS (HR = 2.543; 95% CI 1.224, 5.285; $p = 0.012$; Table II) and DFS (HR = 2.034; 95% CI 1.160, 3.566; $p = 0.013$; Table III). The concordance index (C-index) for OS was 0.713 (95% CI, 0.671, 0.756) and the C-index for DFS was 0.749 (%95 CI 0.729, 0.775). MMP12 expression may thus be a valuable independent prognostic predictor in HCC patients with vascular invasion.

DNA Hypomethylation and MMP12 Overexpression

To gain additional insights into the mechanisms regulating altered MMP12 expression in HCC, we investigated the association between MMP12 expression and methylation. Comparison of the expression of the DNMT1, DNMT3A, and DNMT3B DNA methyltransferases between tumors with high and low levels of MMP12 expression showed that all three DNMTs were upregulated in conjunction with reduced MMP12 expression (Figure 4A). A UALCAN analysis further revealed that DNMT1 was associated

Table I. MMP12 expression and clinicopathological data for individuals in the TCGA-LIHC cohort.

Characteristic	Low expression of MMP12	High expression of MMP12	p
N	187	187	
Etiology, n (%)			
Hepatitis	77 (49.0)	80 (51.0)	0.505
Alcohol consumption	56 (48.7)	59 (51.3)	0.538
Alcohol and hepatitis	23 (47.9)	25 (52.1)	0.512
Other	67 (48.6)	71 (51.4)	0.517
T stage, n (%)			**0.002**
T1	109 (29.4%)	74 (19.9%)	
T2	37 (10%)	58 (15.6%)	
T3	34 (9.2%)	46 (12.4%)	
T4	4 (1.1%)	9 (2.4%)	
N stage, n (%)			0.693
N0	121 (46.9%)	133 (51.6%)	
N1	1 (0.4%)	3 (1.2%)	
M stage, n (%)			0.573
M0	129 (47.4%)	139 (51.1%)	
M1	3 (1.1%)	1 (0.4%)	
Pathologic stage, n (%)			**0.002**
Stage I	102 (29.1%)	71 (20.3%)	
Stage II	35 (10%)	52 (14.9%)	
Stage III	33 (9.4%)	52 (14.9%)	
Stage IV	4 (1.1%)	1 (0.3%)	
Tumor status, n (%)			**0.013**
Tumor free	114 (32.1%)	88 (24.8%)	
With tumor	65 (18.3%)	88 (24.8%)	
Gender, n (%)			**0.047**
Female	51 (13.6%)	70 (18.7%)	
Male	136 (36.4%)	117 (31.3%)	
Age, n (%)			0.055
≤ 60	79 (21.2%)	98 (26.3%)	
> 60	108 (29%)	88 (23.6%)	
BMI, n (%)			0.352
≤ 25	84 (24.9%)	93 (27.6%)	
> 25	85 (25.2%)	75 (22.3%)	
Residual tumor, n (%)			0.588
R0	166 (48.1%)	161 (46.7%)	
R1	8 (2.3%)	9 (2.6%)	
R2	1 (0.3%)	0 (0%)	
Histologic grade, n (%)			**< 0.001**
G1	40 (10.8%)	15 (4.1%)	
G2	102 (27.6%)	76 (20.6%)	
G3	40 (10.8%)	84 (22.8%)	
G4	2 (0.5%)	10 (2.7%)	
Adjacent hepatic tissue inflammation, n (%)			0.179
None	66 (27.8%)	52 (21.9%)	
Mild	47 (19.8%)	54 (22.8%)	
Severe	12 (5.1%)	6 (2.5%)	
AFP (ng/ml), n (%)			**< 0.001**
≤ 400	121 (43.2%)	94 (33.6%)	
> 400	20 (7.1%)	45 (16.1%)	
Albumin (g/dl), n (%)			0.721
< 3.5	35 (11.7%)	34 (11.3%)	
≥ 3.5	125 (41.7%)	106 (35.3%)	
Child-Pugh grade, n (%)			0.646
A	120 (49.8%)	99 (41.1%)	
B	11 (4.6%)	10 (4.1%)	
C	1 (0.4%)	0 (0%)	
Fibrosis ishak score, n (%)			0.739
0	44 (20.5%)	31 (14.4%)	
½	15 (7%)	16 (7.4%)	
¾	14 (6.5%)	14 (6.5%)	
5/6	43 (20%)	38 (17.7%)	

Table continued

Table I *(Continued)*. MMP12 expression and clinicopathological data for individuals in the TCGA-LIHC cohort.

Characteristic	Low expression of MMP12	High expression of MMP12	p
Vascular invasion, n (%)			0.139
No	116 (36.5%)	92 (28.9%)	
Yes	51 (16%)	59 (18.6%)	
OS event, n (%)			**0.039**
Alive	132 (35.3%)	112 (29.9%)	
Dead	55 (14.7%)	75 (20.1%)	
DSS event, n (%)			**0.022**
Alive	153 (41.8%)	134 (36.6%)	
Dead	30 (8.2%)	49 (13.4%)	
PFI event, n (%)			**0.039**
Alive	106 (28.3%)	85 (22.7%)	
Dead	81 (21.7%)	102 (27.3%)	

Table II. Univariate and multivariate Cox proportional hazard analyses of MMP12 expression and overall survival (OS) for patients with HCC in the TCGA-LIHC cohort.

Characteristic	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p	HR (95% CI)	p
Sex (female/male)	0.772 (0.529, 1.127)	0.18		
Age (≤ 60/> 60)	1.228 (0.843, 1.787)	0.285	2.598 (1.417, 4.765)	**0.002**
Tumor stage	1.139 (0.782, 1.657)	0.498		
T stage	2.517 (1.730, 3.661)	< **0.001**		
Vascular invasion	1.322 (0.853, 2.048)	0.212	1.957 (1.030, 3.721)	**0.040**
Tumor Status	1.489 (1.009, 2.197)	**0.045**	2.376 (1.378, 4.096)	**0.002**
AFP	1.3 (0.729, 2.319)	0.374		
MMP12	2.557 (1.715, 3.813)	< **0.001**	2.543 (1.224, 5.285)	**0.012**

Abbreviations: CI, confidence interval; HR, hazard ratio; AFP alpha-fetoprotein. C-index = 0.713 (95% CI 0.671, 0.756).

with a trend of increased methylation in normal liver tissues relative to HCC (p = 0.101, Figure 4B). Further investigation with DiseaseMeth v 2.0 found that MMP12 methylation was significantly reduced in HCC tumors relative to paracancerous tissues (p < 0.0001; Figure 4C). Moreover, two key methylation sites (cg10333701 and cg03604819) in the MMP12 sequence were negatively correlated with MMP12 expression (Figure 4D). Hypermethylated regions were present within the 3'- and 5'-UTR regions, whereas the TSS1500 and TSS200 regions tended to be

Table III. Univariate and multivariate Cox proportional hazard analyses of MMP12 expression and disease-free survival (DFS) for patients with HCC in the TCGA-LIHC cohort.

Characteristic	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p	HR (95% CI)	p
Sex (female/male)	0.835 (0.598, 1.166)	0.290		
Age (≤ 60/> 60)	0.929 (0.665, 1.298)	0.666		
Tumor stage	1.097 (0.798, 1.510)	0.568		
T stage	2.369 (1.696, 3.310)	< **0.001**	2.435 (1.404, 4.222)	**0.002**
Vascular invasion	1.842 (1.281, 2.647)	0.001		
Tumor Status	3.605 (2.602, 4.993)	< **0.001**	5.988 (3.682, 9.738)	< **0.001**
AFP	1.246 (0.764, 2.209)	0.378	2.164 (1.197, 3.912)	**0.011**
MMP12	2.029 (1.415, 2.911)	< **0.001**	2.034 (1.160, 3.566)	**0.013**

Abbreviations: CI, confidence interval; HR, hazard ratio; AFP alpha-fetoprotein. C-index = 0.749 (95% CI 0.729, 0.775).

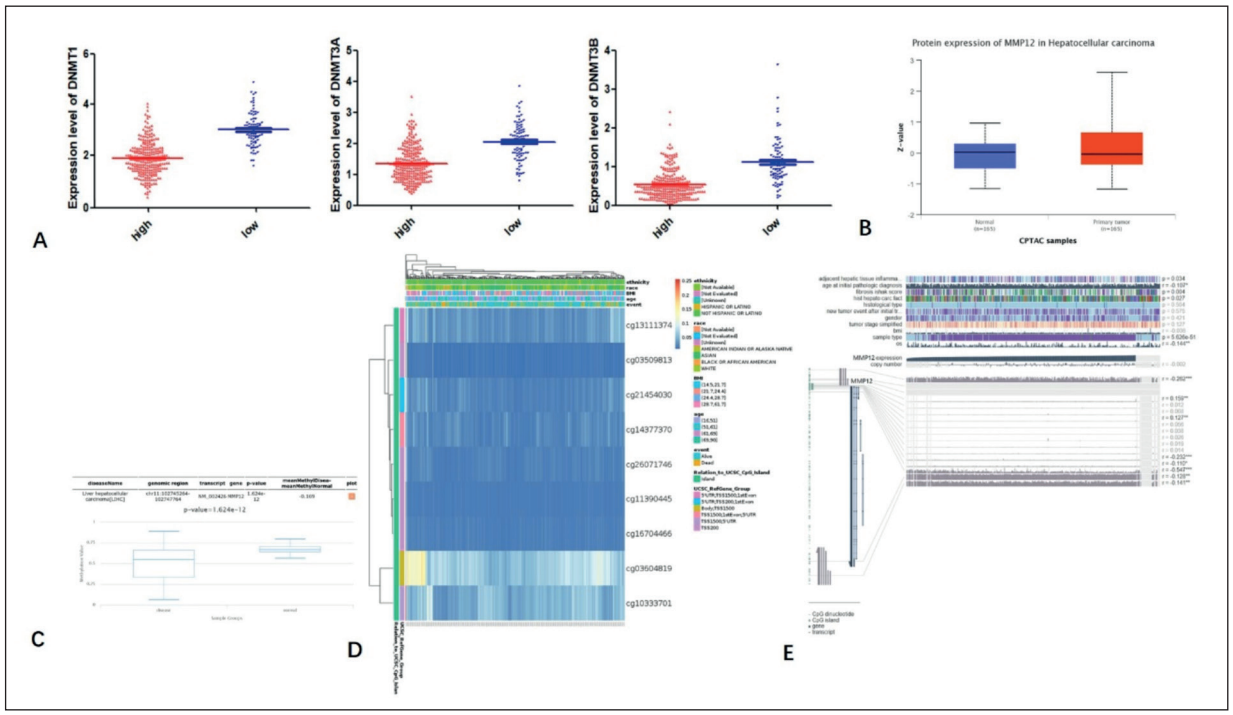

Figure 4. Assessment of the relationship between MMP12 and methylation. **A**, Analysis of the expression of the DNMT1, DNMT3A, and DNMT3B DNA methyltransferases. **B**, Methylation status assessed by UALCAN. **C**, Methylation status assessed by DiseaseMeth v 2.0. **D**, MMP12 DNA methylation and the relationship between methylation and gene expression assessed by MEXPRESS. MMP12 expression levels are represented by blue lines, and the Pearson's correlation coefficient values and *p*-values for particular methylation sites and gene expression levels are shown on the right. **E**, Heatmap showing differentially methylated MMP12 regions, generated by MethSurv.

hypomethylated. The patterns of differential expression of MMP12 expression were visualized using heatmaps that are shown in Figure 4E.

The Association Between Immune Cell Infiltration and MMP12 Expression in HCC

In general, tumor-infiltrating lymphocytes (TILs) are key independent predictors of metastasis and survival outcomes in many types of cancer[17]. We used the TIMER database to explore potential links between MMP12 expression and immune cell infiltration in HCC. An initial SCNA module analysis revealed several infiltrating immune cell populations that were not associated with changes in MMP12 gene copy number in HCC, including CD4+ T cells, DCs, B cells, and macrophages (Figure 5A). We then examined the association between immune cell infiltration and HCC prognosis and showed that increased macrophage infiltration was associated with poorer survival outcomes with an OS of 60 months (p = 0.019, HR=7.117, 95% CI 1.861,27.217, Figure 5B). Gene module analysis showed no correlations between MMP12 expression and tumor purity, while MMP12 expression was positively correlated with the levels of B cells, CD8+ and CD4+ T cells, macrophages, neutrophils, and DCs in HCC (Figure 5C). These findings suggest that MMP12 may impact prognosis and clinical outcomes in HCC by modulating the infiltration of intratumoral immune cells.

To further confirm the link between MMP12 expression and immune cell infiltration, we examined the relationship between MMP12 expression and immunological marker genes associated with the six cell types. This showed that four marker genes (CD19, IRF5, ITGAM, ITGAX) were associated with B cells. Also, M1 macrophages, neutrophils, and DCs were positively correlated with MMP12 expression in the HCC samples (Table IV). These data suggest that the interplay between MMP12 and immune cell populations may impact HCC prognosis.

The Functional Roles of MMP12 in HCC

GO and KEGG enrichment analysis was performed for the top 200 genes mostly strongly correlated with MMP12 expression. This showed

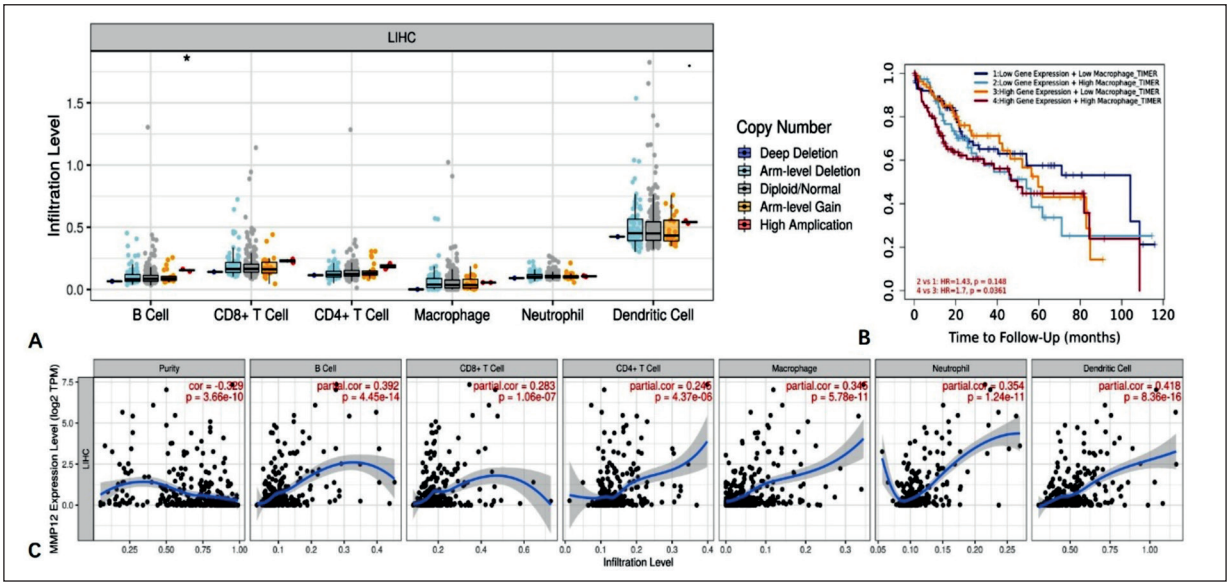

Figure 5. Relationships between MMP12 expression and immune cell infiltration in HCC. **A**, The relationship between MMP12 copy number and immune cell infiltration in HCC. **B**, Associations between immune infiltration and HCC patient OS were assessed with Kaplan-Meier plots. **C**, Correlations between MMP12 expression levels and immune infiltration in HCC. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

that MMP12 was associated with the immune response (Figure 6A-C) in GO and with activation of cytokine-cytokine receptor interaction in KEGG pathways (Figure 6D).

Discussion

In this study, we used *in silico* approaches to show that MMP12 was significantly overex-

Table IV. Correlation analysis between MMP12 and immune cell biomarkers in hepatocellular carcinoma cancer.

Immune cell type	Gene	Correlation Coef.	*p*-value
B cell	**CD19**	**0.26928767**	**1.23E-07**
	CD79A	0.108683427	0.035638416
CD8+ T cell			
	CD8A	0.16540517	0.00134393
	CD8B	0.159708829	0.001947048
CD4+ T cell	CD4	0.164780548	0.001402223
M1 macrophage			
	NOS2	-0.084955461	0.100916288
	IRF5	**0.32337945**	**1.91E-10**
	PTGS2	0.012444166	0.810436917
M2 macrophage			
	CD163	0.030633654	0.554632557
	VSIG4	0.042360396	0.413848183
	MS4A4A	0.029464696	0.569845371
Neutrophil	CEACAM8	0.159715136	0.001946233
	ITGAM	**0.285689954**	**2.16E-08**
	CCR7	0.001159209	0.982167473
Dendritic cell	HLA-DPB1	0.12087236	0.019419822
	HLA-DQB1	0.122723517	0.017626348
	HLA-DRA	0.132122436	0.010578512
	HLA-DPA1	0.08758435	0.090757871
	CD1C	0.054062997	0.297044825
	NRP1	0.124323264	0.016194786
	ITGAX	**0.27742742**	**5.58E-08**

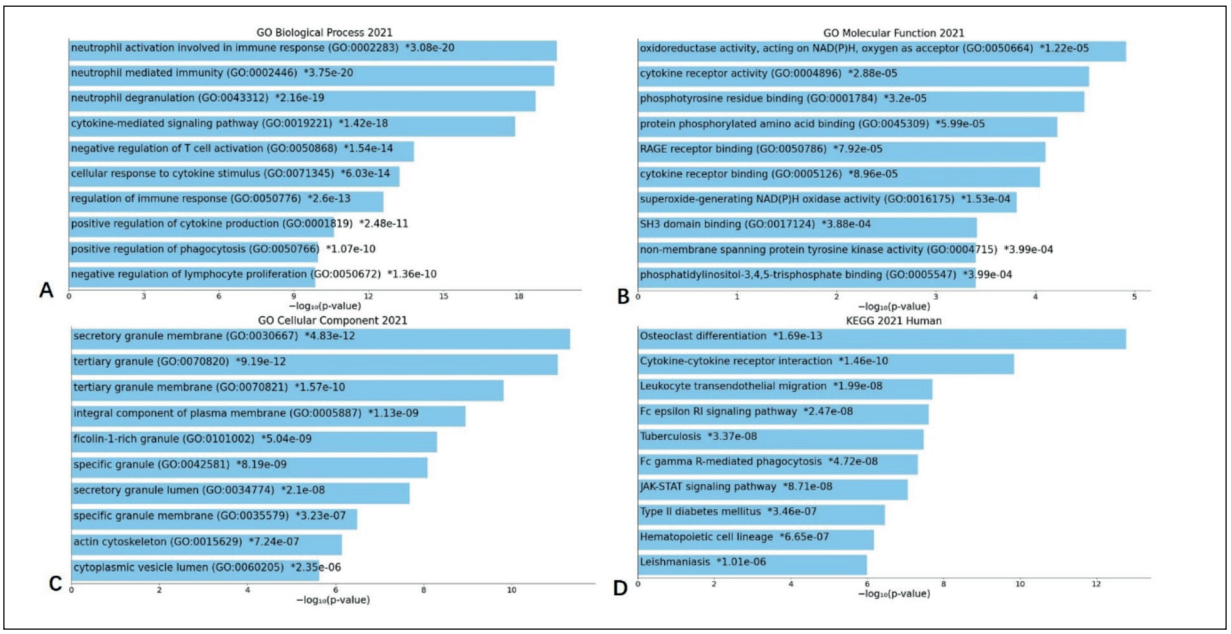

Figure 6. MMP12 functional enrichment analysis. KEGG enrichment and GO analyses of the MMP12-related genes in HCC.

pressed during HCC vascular invasion. We found that VI+ HCC tumors consistently overexpressed MMP12 compared with normal tissues or VI- tumors. Also, we showed that higher levels of MMP12 expression were linked to poor survival outcomes. These data indicate that MMP12 may be a potential therapeutic target and biomarker of vascular invasion in HCC.

Vascular invasion is a primary predictor of metastatic progression, recurrence, and poor survival outcomes in HCC patients[3,4]. Understanding the mechanistic basis of HCC vascular invasion may thus identify novel biomarkers and therapeutic targets to improve patient outcomes[2]. However, relatively few studies have explored mRNAs as prognostic biomarkers of vascular invasion in HCC. The roles of MMP12 have been investigated previously[18-21]. Xu et al[22] showed that MMP12 overexpression is associated with prognosis in gastric cancer making it a promising therapeutic target in this cancer type. Also, Shin et al[23] identified genetic polymorphisms in MMP12 that can function as prognostic biomarkers in breast cancer. Gao et al[24] investigated the importance of MMP12 in the development of HCC.

Alterations in DNA methylation are associated with liver carcinogenesis, whilst DNA methylation and hydroxymethylation have been linked with different outcomes in HCC and cholangiocarcinoma[25,26]. In this study, we found that abnormal MMP12 hypomethylation was associated with overexpression of the gene in HCC tumors in comparison with control tissues. Several DNA methyltransferases, namely, DNMT1, DNMT2, and DNMT3, were consistently upregulated in HCC tissue. Moreover, higher levels of MMP12 expression were associated with increased expression of the three DNA methyltransferases providing a potential mechanism for the upregulation of MMP12 in HCC. Specific methylation sites were also found to be negatively correlated with HCC prognosis. Further investigation of the association between MMP12 expression and genome-wide methylation patterns showed increased hypomethylation proximal to the open sea regions (Figure 4E). These results suggest that abnormal methylation patterns may be associated with poor prognosis in HCC.

Previous work has suggested that immune cell infiltration is a useful prognostic biomarker in cancer patients[27,28]. Analysis using the TIMER database showed that the MMP12 gene copy number was not correlated with the infiltration of B cells, CD4+ and CD8+ T cells, macrophages, neutrophils, and DCs. However, MMP12 expression levels were closely correlated with tumor immune infiltration in HCC with many of the specific cell types associated with prognosis[29]. Positive correlations were observed between MMP12 expression and several immune cell subsets suggesting that MMP12 may regulate

the composition of the tumor immune microenvironment during HCC progression.

To fully clarify the potential role of MMP12 as a regulator of oncogenesis, we performed functional enrichment analyses. Our data showed that MMP12 was associated with the immune pathways.

This study has several limitations. Firstly, all *in silico* analyses of the potential relationships between mRNAs and vascular invasion require further experimental verification. Secondly, the study included relatively small numbers of *in vivo* HCC samples with vascular invasion information. Finally, further verification is required to determine the mechanistic role of MMP12 in HCC.

Conclusions

The results in this study highlight the value of MMP12 as a prognostic biomarker associated with vascular invasion in patients with HCC. The data were from a large TCGA patient dataset and were closely associated with key disease-related parameters. Future research is required to explore the underlying mechanisms through which MMP12 regulates vascular invasion in HCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors' Contribution

Drs. Guo and Jiang proposed the concept and designed the study and Dr. Jiang contributed to the acquisition of data. All authors provided input to the manuscript. All authors read and approved the final manuscript. Conceptualization: Ziyi Guo. Data curation: Ziyi Guo. Formal analysis: Ziyi Guo. Methodology: Lipeng Jiang. Software: Ziyi Guo. Supervision: Lipeng Jiang. Validation: Lipeng Jiang. Writing – original draft: Ziyi Guo, Lipeng Jiang. Writing – review and editing: Ziyi Guo, Lipeng Jiang.

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