Potential Prognostic Biomarkers for Bone Metastasis from Hepatocellular Carcinoma

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Key Words. Gene expression • Tissue microarray • Hepatocellular carcinoma • Bone metastases

Disclosures: Zuo-Lin Xiang: None; Zhao-Chong Zeng: None; Zhao-You Tang: None; Jia Fan: None; Jian He: None; Hai-Ying Zeng: None; Xiao-Dong Zhu: None.

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ABSTRACT

Background. Hepatocellular carcinoma (HCC) most commonly develops in patients who have a viral infection, especially in the case of hepatitis B virus (HBV), and in patients with a chronic liver disease. HCC patients with bone metastasis (BM) suffer from pain and other symptoms that significantly reduce their quality of life. Identification of patients who are at high risk for BM after undergoing potentially curative treatment for HCC remains challenging. Here, we aimed to identify HCC BM-related genes and proteins to establish prediction biomarkers.

Methods. RNA was extracted from 48 pairs of intratumoral and peritumoral formalin-fixed, paraffin-embedded tissue from HCC patients with and without BM. A cDNA-mediated annealing, selection, extension and ligation assay containing 502 cancer-related genes was used to identify novel BM-associated genes. An additional independent study with 350 HCC patients who had undergone hepatectomy was conducted to evaluate the expression of candidate genes at the protein level using immunohistochemistry on tissue microarrays

(TMAs). Of the 350 patients, 273 (78.0%) were infected with HBV.

Results. Seven intratumoral genes and 17 peritumoral genes were overexpressed in patients with BM, whereas 15 intratumoral genes and 28 peritumoral genes were underexpressed in patients with BM. We selected the following four genes for further analysis because they were differentially expressed in the cancer gene-specific microarray and were previously reported to be associated with BM: connective tissue growth factor (CTGF), matrix metalloproteinase-1 (MMP-1), transforming growth factor β 1 (TGF- β 1), and interleukin-11 (IL-11). We assessed the protein expression of these selected genes using immunohistochemistry on TMAs including 350 HCC patient specimens. We determined that expression of intratumoral CTGF, intratumoral IL-11, and peritumoral MMP-1 were independent prognostic factors for developing BM in HCC patients. Combining intratumoral CTGF and IL-11 expression was also an independent risk factor for BM development.

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Conclusions. Sixty-seven genes were differentially expressed in HCC patients with and without BM. High intratumoral CTGF, positive IL-11, and high peritumoral MMP-1 expression were associated with BM after hep-

atectomy. Intratumoral CTGF expression combined with IL-11 expression may serve as a useful predictive biomarker for HCC BM. *The Oncologist* 2011;16:1028–1039

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide in terms of its incidence rate and the third most common cause of cancer-related deaths [1]. In some areas of Asia, HCC is the most common cause of cancer-related deaths. Major interest in this disease has developed worldwide because of its rising incidence in the U.S. and Europe [2, 3]. This disease is very difficult to treat because of its propensity for metastasis and recurrence. The frequency of bone metastasis (BM) in HCC patients with extrahepatic metastases is estimated to be 38.5% [4]. Osteolytic metastases can cause severe pain, pathological fractures, life-threatening hypercalcemia, spinal cord compression, and other nerve-compression syndromes [5]. BM is a common cause of pain and produces other significant symptoms that reduce the quality of life of HCC patients [6]. It is important to develop accurate biomarkers to identify HCC patients at high risk for BM and to develop therapeutic targets to treat these high-risk patients. However, the genes responsible for promoting the interactions between HCC cancer cells and bone are largely unknown.

Formalin-fixed, paraffin-embedded (FFPE) specimens are readily available for research purposes; large numbers are routinely stored by pathology laboratories [7]. The ability to perform gene-expression profiling using these samples should greatly facilitate research attempting to correlate expression profiles with clinical outcomes and will enable both prospective and retrospective studies [8]. The cDNA-mediated annealing, selection, extension and ligation (DASL) assay has been validated for high-throughput expression profiling of archived FFPE clinical samples [9–13]. This approach could be used to discover biomarkers for predicting cancer metastasis and recurrence that could prove to be useful in decisions about therapeutic strategies.

In the present study, we investigated whether variations in gene and protein expression in HCC present at the time of surgery can enable the identification of HCC patients at high risk for developing BM.

MATERIALS AND METHODS

Patients and Tissue Specimens

Forty HCC patients underwent curative resection at Zhongshan Hospital and presented with BM but had no additional

metastases. The matched non-BM (NBM) group included 40 HCC patients who also had no additional metastases after curative resection. The follow-up duration for patients was 5 years. Eighty pairs of intratumoral and peritumoral FFPE HCC tissue specimens were obtained from Zhongshan Hospital between February 1998 and March 2004. Paraffin blocks were selected based only on the suitability of the FFPE tissue and if there were complete clinicopathological and follow-up data for each specimen. Clinicopathological characteristics are summarized in supplemental online Table S1.

An additional study with an independent cohort of 350 HCC patients was conducted to evaluate the clinical significance of the candidate HCC BM genes identified in our preliminary study. The archived specimens were collected by Zhongshan Hospital in January 1999 to March 2004. All patients underwent curative resection for HCC. All patients received a bone scan prior to surgery to exclude BM, and BM was not detected in any of the patients at the time of surgery. Curative resection was defined as complete macroscopic removal of the tumor [14]. This conclusion depended on the presurgical radiological results combined with the postsurgical histology data; curative resection was assigned if the margin of the sample contained no tumor cells. The inclusion criteria were: (a) a curative resection, (b) an HCC diagnosis based on pathology, (c) no prior anticancer treatment, (d) suitable FFPE tissue, and (e) complete clinicopathological and follow-up data. Of the included patients, 273 (78.0%) were infected with hepatitis B virus. Three hundred forty-seven had Child–Pugh class A disease and three patients had Child–Pugh class B disease. Tumors were staged according to the 2002 International Union Against Cancer tumor–node–metastasis (TNM) classification system. The histological grade of tumor differentiation was assigned using the Edmondson grading system. Tumor size was based on the largest dimension of the tumor specimen. Vascular invasion was determined by microscopic examination of the resected specimen. Detailed clinicopathological features are provided in Table 1. Approval for this study was obtained from the Zhongshan Hospital Research Ethics Committee. Informed consent was obtained in accordance with this Committee's regulations.

Table 1. Clinicopathological characteristics of 350 hepatocellular carcinoma patients

Variable	n of patients (%)
Age, yrs	
≤51	169 (48.3)
>51	181 (51.7)
Gender	
Female	50 (14.3)
Male	300 (85.7)
HBsAg	
Negative	77 (22.0)
Positive	273 (78.0)
HCV-Ab	
Negative	344 (98.3)
Positive	6 (1.7)
AFP, ng/mL	
≤20	110 (31.4)
>20	240 (68.6)
ALT, U/L	
≤40	207 (59.1)
>40	143 (40.9)
Liver cirrhosis	
No	44 (12.6)
Yes	306 (87.4)
Child–Pugh class	
A	347 (99.1)
B	3 (0.9)
Tumor differentiation	
I–II	251 (71.7)
III–IV	99 (28.3)
Tumor size, cm	
≤5	172 (49.1)
>5	178 (50.9)
Tumor number	
Single	262 (74.9)
Multiple	88 (25.1)
Tumor encapsulation	
Complete	179 (51.1)
None	171 (48.9)
Vascular invasion	
No	276 (78.9)
Yes	74 (21.1)
TNM stage	
I	251 (71.7)
II–III	99 (28.3)

Tumor differentiation evaluation was based on Edmondson–Steiner grading.

Abbreviations: AFP, α -fetoprotein; ALT, alanine aminotransferase; HBsAg, hepatitis B virus surface antigen; HCV-Ab, hepatitis C virus antibody; TNM, tumor–node–metastasis.

Follow-Up

The follow-up cutoff time was March 2009, and the median follow-up time was 53.9 months (range, 3.0–120.6 months). We previously published our follow-up and BM diagnostic procedures [6, 15, 16]. Briefly, follow-up was performed at 3-month intervals after hepatectomy. At every visit, a medical history was recorded and physical examination was performed. Chest radiography was performed every 6 months. Ultrasonographic examination of the liver and lymph nodes of the abdomen as well as laboratory tests were performed every 3 months. A bone scan was performed on a yearly basis. Bone scanning or magnetic resonance imaging was immediately performed if there was any report of localized bone pain. A diagnosis of BM was based on a history of HCC, the presence of symptoms, and radiological imaging studies. The time interval between the date of surgery and the date of presentation of BM was recorded. Treatment modalities after relapse were administered according to previously described guidelines [6, 17, 18]. Briefly, when a diagnosis of BM was made, external beam radiotherapy was focused on the involved bone. Other site relapses received radiotherapy, interventional therapy, or surgery.

RNA Isolation

RNA was extracted from the tissue of 40 pairs of HCC patients with BM and NBM patients. In total, 160 FFPE tissue specimens of intratumoral and matched peritumoral tissue samples were collected from these patients. For total RNA isolation from FFPE tissue, hematoxylin and eosin–stained slides were screened to identify the optimal intratumoral and peritumoral tissue to use for analysis. Two to four 1-mm cores were removed from each block, placed in 800 μ L of Hemo-De, and mixed several times. We used the High Pure RNA Paraffin Kit (Roche Diagnostics GmbH, Penzberg, Germany). Proteinase K digestion was performed for 12 hours. All purification, DNase treatment, and additional steps were performed according to the manufacturer's protocol. RNA samples were stored at -80°C until use.

Quality Assessment of FFPE RNA

We converted 200 ng of RNA to cDNA using 5 μ L of the RNA template. To polymerase chain reaction (PCR) amplify the *RPL13a* gene, we used 500 nM of the forward primer (5'-GTACGCTGTGAAGGCATCAA-3') and reverse primer (5'-GTTGGTGTTCATCCGCTTG-3') and 25 μ L of SYBR Green PCR Master Mix (Applied Biosystems Inc, Foster City, CA). The total PCR volume was 50 μ L. PCR amplifications consisted of an initial enzyme activation step at 95°C for 12 minutes, followed by 40 cycles of 95°C for 20 seconds, 54°C for 20 seconds, and 72°C for

60 seconds. The RNA sample with a cycle threshold (Ct) value ≤ 28 was considered to be of sufficient quality for use in the DASL assay.

DASL Assay

cDNA microarray hybridization was performed to identify differentially expressed genes in intratumoral and peritumoral specimens from BM and NBM HCC patients. The data were normalized using the GenomeStudio Gene Expression Module (version 1.5.4; Illumina Inc., San Diego, CA). Three different probes were designed for each gene, and the three measurements were averaged. The Human Cancer Panel DASL Assay (Illumina Inc.), which includes 502 known cancer-related genes, was used for the DASL assay following the manufacturer's instructions [9]. Images were extracted and fluorescence intensities were read on a BeadArray™ Reader (Illumina Inc.). The scanned data were uploaded into GenomeStudio (Illumina Inc.) for further analysis. Because the DASL Human Cancer Panel supports 96 samples at most, we selected the intratumoral and peritumoral tissue specimens of 48 patients (total, 96 samples) to analyze with this assay.

Tissue Microarray

A tissue microarray (TMA) was constructed as previously described [19]. Hematoxylin and eosin-stained slides were screened to identify the optimal intratumoral and peritumoral tissue to use for analysis. TMA slides were then constructed (in collaboration with Shanghai Biochip Company, Ltd., Shanghai, China) using another independent cohort of 350 HCC patients. Two cores of tissue were collected from non-necrotic areas of tumor foci and from peritumoral tissue adjacent to the tumor. To assess the possibility that positive expression reflected a direct effect of the tumors, we took peritumoral tissue within 10 mm. Punch cores with a longest dimension of 1.0 mm were used. TMA sections with 350 pairs of intratumoral and matched peritumoral samples were constructed. Sections (4 μ m) of the resulting TMA blocks were prepared using standard techniques.

Immunohistochemistry

The immunohistochemistry protocols were described previously [19]. Primary antibodies were mouse anti-human monoclonal antibody for connective tissue growth factor (CTGF), metalloproteinase (MMP)-1, and transforming growth factor (TGF)- β 1 and rabbit anti-human polyclonal antibody for interleukin (IL)-11 (Santa Cruz Biotechnology, Santa Cruz, CA). TMA slides were incubated with primary antibodies overnight at 4°C and washed to remove excess primary antibody. The Envision-plus system (EnVision+HRP/Mo, Dako, Carpinteria, CA) was used for

detection. Reaction products were visualized by incubation with 3,3'-diaminobenzidine. Sections were dehydrated, counterstained with hematoxylin, and mounted. Negative controls were identically treated, but the primary antibody incubation step was omitted.

Immunohistochemistry Scoring

Positive staining was quantified using a computerized imaging system composed of a Leica charge-coupled device camera (DFC420) connected to a Leica DM IRE2 microscope (Leica Microsystems Imaging Solutions Ltd., Cambridge, U.K.). Under 400 $\times$ magnification, images from 10 representative fields were captured using Leica QWin Plus software (version 3; Leica Microsystems Imaging Solutions Ltd.). Identical settings were used for each image. Immunohistochemical staining was assessed in an outcome-blinded fashion by three authors using a compound microscope. We randomly selected 10 high-power fields (magnification, $\times 400$; 100 cells/high-power field) and counted 1,000 cells in each core [20]. The percentage of positive tumor cells was determined by each observer, and the average of the three scores was calculated. In this study, the percentages of positive cells expressing CTGF, TGF- β 1, IL-11, and MMP-1 were categorized as follows: $< 50\%$ (low) and $\geq 50\%$ (high) for CTGF [21], $< 5\%$ (–) and $\geq 5\%$ (+) for TGF- β 1 [22], $< 10\%$ (–) and $\geq 10\%$ (+) for IL-11 [23], and $< 10\%$ (low) and $\geq 10\%$ (high) for MMP-1 [24].

Statistical Analysis

Statistical analyses were performed with SPSS 16.0 software (SPSS Inc., Chicago, IL). The Fisher's exact test was used to compare qualitative variables, and quantitative variables were analyzed by Pearson's correlation test. Logistical regression analyses were used to determine the risk factors of the parameters. Potential association between expression of the candidate molecule and a higher incidence of BM was analyzed as censored time-to-event data using a log-rank test and Cox regression model. A two-tailed $p < .05$ was considered significant. The p -values of the differential gene expression analysis were obtained with Illumina GenomeStudio software, using the "Illumina custom" error model.

RESULTS

Preparation of RNA Samples for DASL

RNA was successfully isolated from all 160 (100%) FFPE specimens. Quantitative PCR confirmed that 129 of these (80.6%) samples were suitable for DASL (Ct ≤ 28). These RNA samples included 30 pairs of intratumoral and peritumoral tissue from the HCC BM group and 26 pairs of intra-

Table 2. Clinicopathological characteristics of 24 HCC patients with BM and 24 matched HCC patients without BM used for DASL assay

Variable	n of patients		p-value
	BM (n = 24)	NBM (n = 24)	
Age, yrs			
≤51	13	14	1.000
>51	11	10	
Gender			
Female	4	4	1.000
Male	20	20	
HBsAg			
Negative	3	4	1.000
Positive	21	20	
HCV-Ab			
Negative	23	24	1.000
Positive	1	0	
AFP, ng/mL			
≤20	9	7	.760
>20	15	17	
ALT, U/L			
≤40	14	13	1.000
>40	10	11	
Liver cirrhosis			
No	4	4	1.000
Yes	20	20	
Child–Pugh class			
A	24	24	NA
B	0	0	
Tumor differentiation			
I–II	14	17	.547
III–IV	10	7	
Tumor size, cm			
≤5	10	13	.564
>5	14	11	
Tumor number			
Single	14	16	.766
Multiple	10	8	
Tumor encapsulation			
Complete	12	13	1.000
None	12	11	
Vascular invasion			
No	13	16	.556
Yes	11	8	

(continued)

Table 2. (Continued)

	<i>n</i> of patients		
Variable	BM (<i>n</i> = 24)	NBM (<i>n</i> = 24)	<i>p</i> -value
TNM stage			
I	15	19	.341
II–III	9	5	
Tumor differentiation evaluation was based on Edmondson–Steiner grading. Abbreviations: AFP, α -fetoprotein; ALT, alanine aminotransferase; BM, bone metastasis; DASL, cDNA-mediated annealing, selection, extension and ligation; HBsAg, hepatitis B virus surface antigen; HCC, hepatocellular carcinoma; HCV-Ab, hepatitis C virus antibody; NA, not available; NBM, no bone metastasis; TNM, tumor–node–metastasis.			

tumoral and peritumoral tissue from the HCC NBM group. The remaining 17 samples included: two intratumoral tissues from the HCC BM group and eight intratumoral tissues from the HCC NBM group, and four peritumoral tissues from the HCC BM group and three peritumoral tissues from the HCC NBM group. To eliminate the impact of clinicopathological factors (excluding BM) on gene-expression profiles, we matched the BM and NBM groups so that there were no significant differences in any clinicopathological feature except BM. We selected 24 pairs from 30 pairs of intratumoral and peritumoral tissue RNA samples in the BM group and 24 pairs from 26 pairs of intratumoral and peritumoral tissue RNA samples in the NBM group. For these 24 BM patients, surgery occurred in May 1999 to February 2004. The median time from surgery to the appearance of bone metastasis was 9.9 months (range, 2.0–60.8 months). Therefore, in total, 48 pairs (96 samples) from 48 HCC patients were subjected to DASL. Detailed clinicopathological features are provided in Table 2. As shown in supplemental online Table S2, tissues from these 48 patients exhibited more vascular invasion ($p = .010$) than tissues from the cohort of 350 patients who were analyzed to evaluate protein expression of the candidate genes.

Testing DASL Assay Performance

Sixty-seven genes were identified as being differentially expressed in the BM and NBM groups (supplemental online Table S3). Seven genes were overexpressed and 17 genes were underexpressed in the intratumoral tissue from the BM group, compared with the NBM group. Fifteen genes were overexpressed and 28 genes were underexpressed in the peritumoral tissue from the BM group, compared with the NBM group. (Heatmaps are shown in supplemental online Figure S1.) (Microarray data are available at <http://www.ncbi>.

Figure 1. CTGF, TGF- β 1, IL-11, and MMP-1 expression. Representative CTGF, TGF- β 1, IL-11, and MMP-1 staining. (A): High intratumoral expression of CTGF. (B): Low intratumoral expression of CTGF. (C): High peritumoral expression of CTGF. (D): Low peritumoral expression of CTGF. (E): Positive intratumoral expression of TGF- β 1. (F): Negative intratumoral expression of TGF- β 1. (G): Positive peritumoral expression of TGF- β 1. (H): Negative peritumoral expression of TGF- β 1. (I): Positive intratumoral expression of IL-11. (J): Negative intratumoral expression of IL-11. (K): Positive peritumoral expression of IL-11. (L): Negative peritumoral expression of IL-11. (M): High intratumoral expression of MMP-1. (N): Low intratumoral expression of MMP-1. (O): High peritumoral expression of MMP-1. (P): Low peritumoral expression of MMP-1. Bar, 50 μ m.

Abbreviations: CTGF, connective tissue growth factor; IL-11, interleukin-11; MMP-1, matrix metalloproteinase-1; TGF- β 1, transforming growth factor- β 1.

nlm.nih.gov/geo/query/acc.cgi?acc=GSE27635; accession number, GSE27635.) From those genes that were most highly overexpressed in the BM group, we selected *CTGF* [25–29], *IL-11* [25–29], *MMP-1* [25–27, 29], and *TGF- β 1* [30, 31] based on a literature review. To determine whether these four potential biomarkers can predict BM in HCC, we performed immunohistochemistry on TMAs that included 350 HCC patient specimens.

Expression of Potential Biomarkers in TMA

CTGF, TGF- β 1, IL-11, and MMP-1 were mainly localized to the cytoplasm of tumor cells or hepatocytes (Fig. 1). IL-11 was also localized to some nuclei. TGF- β 1 and MMP-1 were also partly expressed in fibroblastic cells. The median percentage of cells positive for intratumoral expres-

sion was 36.4% (interquartile range [IQR], 13.7%–69.3%) for CTGF, 6.9% (IQR, 2.6%–12.8%) for IL-11, 5.3% (IQR, 0.9%–11.1%) for TGF- β 1, and 7.8% (IQR, 3.4%–13.4%) for MMP-1. The median percentage of cells positive for peritumoral expression was 10.6% (IQR, 4.1%–52.8%) for CTGF, 4.8% (IQR, 1.1%–12.1%) for IL-11, 1.9% (IQR, 0.2%–5.8%) for TGF- β 1, and 8.7% (IQR, 3.9%–16.1%) for MMP-1. Of the 350 patients, 64 (18.3%) had high intratumoral CTGF expression and 31 (8.9%) had high peritumoral CTGF expression. For IL-11, 92 (26.3%) patients had positive intratumoral expression and 67 (19.1%) patients had positive peritumoral expression. For TGF- β 1, 196 (56.0%) patients had positive intratumoral expression and 19 (5.4%) patients had positive peritumoral expression. For MMP-1, 142 (40.6%) patients had high intratumoral

Figure 2. Kaplan–Meier and log-rank analysis of bone metastasis ratios during the follow-up time for intratumoral CTGF (A), intratumoral IL-11 (B), intratumoral TGF- β 1 (C), and peritumoral MMP-1 (D).

Abbreviations: CTGF, connective tissue growth factor; IL-11, interleukin-11; MMP-1, matrix metalloproteinase-1; TGF- β 1, transforming growth factor- β 1.

expression and 153 (43.7%) patients had high peritumoral expression.

Correlations Between CTGF, TGF- β 1, IL-11, and MMP-1 Expression and Clinicopathological Features

As shown in supplemental online Table S4, patients with high intratumoral CTGF expression were more likely to exhibit high rates of vascular invasion ($p = .041$). Those with high intratumoral IL-11 expression were more likely to be at a higher TNM stage ($p = .043$). Peritumoral CTGF ($p = .013$) and MMP-1 ($p < .001$) overexpression tended to correlate with a lack of tumor encapsulation.

Correlation analysis among these four potential biomarkers revealed that intratumoral CTGF and IL-11 correlated with high intratumoral TGF- β 1 expression: $r = 0.285$ ($p < .001$) and $r = 0.202$ ($p < .001$), respectively.

At last follow-up, 104 (29.7%) patients presented with extrahepatic metastases, including 41 (11.7%) with BM. To eliminate the effect of other extrahepatic metastases on the expression of the four potential biomarkers, we further investigated their expression in the subgroup exhibiting extrahepatic metastases. Correlations between extrahepatic metastases and expression of the four proteins are provided in supplemental

online Table S5. Patients with high intratumoral CTGF and IL-11 expression were more likely to exhibit high rates of BM ($p = .001$ and $p = .034$, respectively). In addition, high peritumoral MMP-1 expression was also associated with a high rate of BM ($p = .002$). There was no relationship identified between expression of the four proteins and lung, adrenal gland, or lymph node metastasis.

Log-Rank Test and Cox Regression Analysis of Potential Biomarkers and BM

As shown in Figure 2A, a log-rank test indicated that high intratumoral CTGF expression was associated with a higher incidence of BM than with low CTGF expression ($p < .001$). Patients with positive intratumoral IL-11 expression had a higher incidence of BM than patients negative for IL-11 expression ($p = .005$) (Fig. 2B). Patients with positive intratumoral TGF- β 1 expression also had a higher incidence of BM than patients negative for TGF- β 1 expression ($p = .039$) (Fig. 2C). However, there was no difference in the incidence of BM between patients with high intratumoral expression of MMP-1 and those with low expression ($p = .245$). In terms of peritumoral tissue, there was no difference in the incidence of BM for patients with high versus low CTGF expression ($p = .152$), patients who were positive versus negative for IL-11 expres-

Table 3. Univariate analyses of factors associated with bone metastasis in a cohort of 350 hepatocellular carcinoma patients

Variable	Bone metastasis		
	HR	95% CI	p-value
Age, yrs (≤ 51 versus > 51)	0.769	0.416–1.421	.402
Gender (female versus male)	1.227	0.481–3.129	.669
HBsAg (negative versus positive)	1.432	0.635–3.230	.387
HCV-Ab (negative versus positive)	1.459	0.200–10.630	.709
AFP, ng/mL (≤ 20 versus > 20)	0.848	0.449–1.602	.612
ALT, U/L (≤ 40 versus > 40)	1.163	0.627–2.155	.632
Liver cirrhosis (no versus yes)	2.884	0.696–11.943	.144
Child-Pugh score (A versus B)	0.049	0.000–3.178	.706
Tumor differentiation (I–II versus III–IV)	2.213	1.194–4.105	.012
Tumor size, cm (≤ 5 versus > 5)	0.990	0.536–1.827	.975
Tumor number (single versus multiple)	2.116	1.128–3.970	.020
Tumor encapsulation (complete versus none)	1.158	0.631–2.125	.635
Vascular invasion (no versus yes)	3.758	2.031–6.953	$< .001$
TNM stage (I versus II–III)	3.155	1.706–5.836	$< .001$
Intratumoral			
CTGF (low versus high)	3.585	1.925–6.679	$< .001$
TGF- $\beta 1$ (negative versus positive)	2.006	1.023–3.934	.043
IL-11 (negative versus positive)	2.351	1.268–4.361	.007
MMP-1 (low versus high)	0.679	0.351–1.310	.248
Peritumoral			
CTGF (low versus high)	2.250	0.997–5.076	.051
TGF- $\beta 1$ (negative versus positive)	1.323	0.408–4.287	.641
IL-11 (negative versus positive)	1.304	0.622–2.733	.482
MMP-1 (low versus high)	3.811	1.910–7.605	$< .001$

Tumor differentiation evaluation was based on Edmondson-Steiner grading.
 Univariate analysis and Cox proportional hazards regression model.
 Abbreviations: AFP, α -fetoprotein; ALT, alanine aminotransferase; CI, confidence interval; CTGF, connective tissue growth factor; HBsAg, hepatitis B virus surface antigen; and HCV-Ab, hepatitis C virus antibody; HR, hazard ratio; IL-11, interleukin 11; MMP-1, matrix metalloproteinase 1; TGF- $\beta 1$, transforming growth factor $\beta 1$; TNM, tumor–node–metastasis.

sion ($p = .480$), or patients who were positive versus negative for TGF- $\beta 1$ expression ($p = .639$). However, as shown in Figure 2D, patients with high peritumoral expression of MMP-1 had a higher incidence of BM than patients with low expression of peritumoral MMP-1 ($p < .001$).

As shown in Table 3, Cox regression univariate analysis showed that tumor differentiation ($p = .012$), tumor number ($p = .020$), vascular invasion ($p < .001$), TNM stage ($p < .001$), intratumoral CTGF expression ($p < .001$), intratumoral TGF- $\beta 1$ expression ($p = .043$), intratumoral IL-11 expression ($p = .007$), and peritumoral MMP-1 expression ($p < .001$) were associated with BM. Multivariate Cox proportional hazards analyses revealed that tumor number ($p = .046$), tumor differentiation ($p = .002$), vascular invasion ($p = .002$), TNM

stage ($p = .005$), intratumoral CTGF expression ($p = .021$), intratumoral IL-11 expression ($p = .007$), and peritumoral MMP-1 expression ($p = .002$) were independent prognostic factors for developing BM in HCC patients (Table 4).

BM Predictors

Logistical regression analysis identified vascular invasion (odds ratio [OR], 2.755; 95% confidence interval [CI], 1.322–5.740; $p = .007$), TNM stage (OR, 2.564; 95% CI, 1.257–5.229; $p = .010$), intratumoral CTGF expression (OR, 3.324; 95% CI, 1.580–6.993; $p = .002$), intratumoral IL-11 expression (OR, 2.401; 95% CI, 1.170–4.924; $p = .017$), peritumoral MMP-1 expression (OR, 4.124; 95% CI, 1.993–8.536; $p < .001$), and intratumoral CTGF combined

Table 4. Multivariate analyses of factors associated with bone metastasis in a 350 hepatocellular carcinoma patient cohort

Variable	HR	95% CI	p-value
Tumor number (single versus multiple)	2.004	1.011–3.971	.046
Tumor differentiation (I–II versus III–IV)	2.833	1.473–5.446	.002
Vascular invasion (no versus yes)	2.760	1.439–5.295	.002
TNM stage (I versus II–III)	2.483	1.318–4.675	.005
Intratumoral CTGF (low versus high)	2.397	1.141–5.036	.021
Intratumoral TGF- β 1 (negative versus positive)	0.868	0.399–1.889	.721
Intratumoral IL-11 (negative versus positive)	2.533	1.295–4.955	.007
Peritumoral MMP-1 (low versus high)	2.973	1.468–6.021	.002

Variables were adopted for their prognostic significance on univariate analysis.

Tumor differentiation evaluation was based on the Edmondson-Steiner grading.

Multivariate analysis and Cox proportional hazards regression model.

Abbreviations: CI, confidence interval; CTGF, connective tissue growth factor; HR, hazard ratio; IL-11, interleukin-11; MMP-1, matrix metalloproteinase 1; TGF- β 1, transforming growth factor β 1; TNM, tumor–node–metastasis.

with IL-11 expression (low intratumoral CTGF expression and negativity for IL-11 expression versus high CTGF expression and/or positivity for IL-11 expression; OR, 3.180; 95% CI, 1.616–6.259; $p = 0.001$) as independent risk factors for BM. We analyzed whether or not there was a correlation between expression of the proteins and BM. We determined that intratumoral CTGF ($r = 0.218$; $p < .001$), intratumoral IL-11 ($r = 0.206$; $p < .001$), and peritumoral MMP-1 ($r = 0.216$; $p < .001$) expression were correlated with BM.

DISCUSSION

HCC patients with BM not only have a poor prognosis but also suffer from pain and other symptoms that greatly reduce their quality of life. We previously reported on the poor prognosis of HCC patients with BM even after these patients undergo radiotherapy [6]. If HCC patients who are at high risk for developing BM were identified when hepatectomy was performed, we could potentially take measures to prevent the development of BM. Therefore, it is important to find biomarkers to predict BM after patients undergo curative resection. Primary tumors may already have a gene-expression profile that is strongly predictive of metastasis and poor survival [32]. Ye et al. [33] previously suggested that genes favoring metastasis progression are present in primary HCC tumors. However, other studies reported that HCC metastases and prognosis are based on a reproducible gene-expression signature in the liver microenvironment adjacent to the primary tumor [11, 34]. This is why we collected both intratumoral and peritumoral tissue from HCC patients.

The biology underlying the bone-specific metastasis of HCC is poorly understood. In this study, we searched

for genes that were expressed in primary tumors that preferentially metastasize to the bone. We identified 67 genes that were differentially expressed between HCC patients with BM and NBM HCC patients. We selected four of the most highly overexpressed genes for further analysis—*CTGF*, *TGF- β 1*, *IL-11*, and *MMP-1*. Expression of these four genes was analyzed in an independent cohort study with 350 HCC patients. We found that high intratumoral CTGF expression, positive intratumoral IL-11 expression, and high peritumoral MMP-1 expression were independent risk factors for developing BM after resection of the primary tumor. The combination of intratumoral CTGF and IL-11 protein expression was better at predicting HCC BM.

CTGF is a member of the CCN family; it is a secreted growth factor that can bind to integrins expressed on the cell surface. CTGF has a broad range of in vivo functions, including proliferation, migration, and angiogenesis [35]. Interestingly, *CTGF* has been identified as an oncogene in a variety of cancer types but is considered a tumor-suppressor gene in other cancer types. CTGF overexpression has been associated with the progression of HCC [36], pancreatic cancer [37], esophageal adenocarcinoma [38], glioblastoma [39], and adult acute lymphoblastic leukemia [40]. In contrast, CTGF overexpression is also associated with the suppression of metastasis and is a favorable prognostic marker in gallbladder cancer [41], lung adenocarcinoma [42], and colorectal cancer [21]. In the present study, we determined that intratumoral CTGF overexpression was associated with BM in HCC patients. This is consistent with previous studies demonstrating that CTGF overexpression is associated with BM in breast cancer patients and may be a BM-specific biomarker [25, 27].

IL-11 belongs to the IL-6 cytokine family; it was initially described as a bone marrow stroma-derived hemopoietic cytokine and a potent stimulator of osteoclast formation [43]. It is known to enhance bone resorption by promoting osteoclastogenesis and by suppressing the activity of osteoblasts [44]. IL-11 promotes the development of gastric cancer [45], breast cancer [46], and colorectal cancer [47]. We determined that overexpression of intratumoral IL-11 correlated with BM, which is consistent with the reported role of IL-11 in BM in breast cancer patients [27, 48]. This study is the first to report a relationship between HCC BM and IL-11 expression. We previously reported that metastasis of HCC to the bone was predominately osteolytic [6]. It is possible that IL-11 plays an important role in this process.

CTGF and IL-11 are known targets of TGF- β signaling because their expression is induced via activation of the canonical TGF- β /Smad pathway. These cytokines may participate in a bone metastasis cycle driven by paracrine TGF- β signaling [25]. In the present study, we also determined that expression of CTGF and IL-11 correlated with TGF- β 1 expression. This suggests that overexpression of TGF- β 1 may cause a further increase in the expression of downstream metastasis genes to establish a positive feedback metastasis cycle [25]. Maintenance of IL-11 and CTGF expression in these cells would increase their osteolytic bone-metastatic activity.

We previously reported that overexpression of chemokine receptor 4 (CXCR4) by HCC tumor cells was associated with HCC BM [16]. The HCC tumor cell may signal through CXCR4/stromal cell-derived factor 1 to promote homing to the bone microenvironment. Greater expression of CTGF and IL-11 coupled with their further induction by bone-derived TGF- β would favor tumor growth in the bone. This would result in a CTGF-IL-11-TGF- β 1 positive feedback metastasis cycle, which would stimulate potent BM in HCC. As hypothesized by the seed and soil theory of Paget, the molecular interaction of circulating cancer cells with the bone microenvironment is critical for BM [49]. This study identified that bone-specific metastasis by HCC cells was associated

with BM-specific genes and proteins, including IL-11 and CTGF.

In the present study, we determined that overexpression of CTGF and IL-11 in tumor tissue, but not in the peritumoral liver tissue, was associated with BM. But peritumoral overexpression of MMP-1 in the BM versus NBM group was also an independent risk factor for BM in the independent cohort study of 350 HCC patients. This finding coincides with a previous report that MMP-1 may be related to BM [25–27]. It may be that the intratumoral and peritumoral microenvironments may differentially contribute to BM for HCC patients. Our findings demonstrated that intratumoral CTGF expression combined with IL-11 may be useful in predicting the development of BM in HCC. Once HCC patients who are at a high risk for BM are identified, the physician may wish to include bisphosphonates as an additional adjuvant systemic therapy in order to prevent BM. Our study identified potential genes and proteins that participate in the process of HCC metastasis to bone; however, a detailed mechanism of how HCC tumors cells target and colonize bone has yet to be established.

ACKNOWLEDGMENTS

We thank Wei-Zhong Wu and Dong-Mei Gao (Liver Cancer Institute, Zhongshan Hospital) for their expert technical assistance.

This study was sponsored by the National Nature Science Foundation of China (grant no. 30973500).

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This study was conducted with the approval of Zhongshan Hospital Research Ethics Committee.

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