

## Original Article

# Perineural invasion: a potential reason of hepatocellular carcinoma bone metastasis

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**Abstract:** The nervous system plays an important role in the regulation of epithelial homeostasis and has also been postulated to play a role in tumorigenesis. Perineural invasion (PNI) is the only interaction between cancer cells and nerves studied to date. It is a symbiotic relationship between cancer cells and nerves that result in growth advantage for both. The potential association between HCC bone metastases and PNI is unknown. In this study, we investigate the nerve density in HCC and paired bone metastases to reveal the potential association of HCC bone metastases and PNI. The nerve density was evaluated by immunohistochemistry in formalin-fixed paraffin embedded (FFPE) hepatocellular carcinoma (HCC) and paired bone metastases tissues from 13 HCC patients with synchronous or metachronous bone metastases that underwent surgical resection. FFPE specimens of HCC bone metastases tissues express higher perineural density than HCC tissues, pointing to a potential role of the PNI in bone metastases from HCC. This is the first description of the potential association of PNI and HCC bone metastases.

**Keywords:** Hepatocellular carcinoma, bone metastasis, perineural invasion, nerve density

## Introduction

Liver cancer in men is the fifth most frequently diagnosed cancer worldwide but the second most frequent cause of cancer death. In women, it is the seventh most commonly diagnosed cancer and the sixth leading cause of cancer death. Half of these cases and deaths were estimated to occur in China [1]. Among primary liver cancers, hepatocellular carcinoma (HCC) represents the major histological subtype, accounting for 70% to 85% of the total liver cancer burden worldwide [1]. The primary risk factor for HCC is liver injury from diverse causes that leads to hepatic cirrhosis in most patients. An estimated 78% of HCC cases and 57% of cases of liver cirrhosis are caused by chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) [2, 3]. HCC is the sixth most prevalent cancer worldwide and the third leading cause of cancer-related death, although its geographical distribution is heterogeneous with the highest incidence in sub-Saharan

Africa and Eastern Asia [4]. Bone is an uncommon site of metastasis in HCC, with the incidence ranging from 3% to 20% [5]. Although bone involvement is reported as uncommon in HCC, its incidence has significantly increased in the last decade due to the longer survival of HCC patients related to recent progresses made both in the diagnosis and treatment of the disease [5-7].

A better understanding of the pathogenic mechanisms underlying the spread of bone metastases in HCC is important. Some retrospective studies have described the characteristics of bone metastasis from HCC [5, 8-10]. However, few data are yet available about bone involvement in patients with HCC, and no agreement has yet been reached about the treatment strategy for extrahepatic HCC metastases. The nature and the characteristics of bone metastases in HCC have not been fully explored in literature, presumably because HCC skeletal involvement was rarely diagnosed.

There is crosstalk between tumor cells and nerves, such that tumors induce active neurogenesis, resulting in increased neuronal density in preneoplastic and neoplastic tissues [11-13]. Prior studies have described a process termed perineural invasion (PNI) in which tumor cells grow and migrate along native nerve fibers. This process is associated with a poor prognosis, possibly because the nerves provide survival signals to the tumors [14] or provide a gateway toward hematogenous spread [15]. PNI is a frequent clinical and pathological finding in head and neck, pancreatic, prostate, and other cancers [15], and has been shown to be a marker of poor outcome [16]. Early studies have suggested that PNI involves signaling amongst tumor, nerve, and stromal cells through paracrine mechanisms [17, 18]. Neurotrophic and axonal guidance molecules have potent effects on axonal growth, and may be upregulated in cancers with a predilection for nerve invasion [19-21].

The potential association between HCC bone metastases and PNI is unknown, partially because the tumor tissues of HCC and paired BM are not easy to obtain. In this study, we investigate the nerve density in HCC and paired bone metastases to reveal the potential association of HCC bone metastases and PNI.

## Patients and methods

### *Patients and tumor tissue samples*

The institutional ethical committee approved the current retrospective study. A written informed consent was obtained for all patients. We reviewed the electronic medical records of consecutive patients in whom HCC and synchronous or metachronous bone metastasis was newly diagnosed from January 2009 to October 2014 at the Department of Orthopedics, the First Affiliated Hospital, Zhejiang University School of Medicine (**Table 1**). The diagnosis of HCC was mainly based on recommendations of the American Association for the Study of Liver Diseases [22]. All patients underwent blood investigations, which included complete blood count, liver function tests, and tests for viral markers of hepatitis B and C infection. Serum alpha-fetoprotein (AFP) was estimated using a particle enzyme immunoassay (AxSYM System; Abbott Laboratories, Abbot Park, Illinois, USA; normal value <20 ng/ml). Upper gastrointestinal endoscopy was done in each

case to detect the presence of esophageal varices. Patients with underlying cirrhosis were classified into Child's A, B or C based on the Child-Pugh classification [23]. Staging of HCC was done based on the Barcelona Clinic Liver Cancer (BCLC) staging protocol [24]. All patients were evaluated pre-operatively using abdominal computed tomographic (CT) scan, magnetic resonance imaging (MRI), or fluorine-18 fluoro-deoxyglucose positron emission tomography/computed tomographic scan (<sup>18</sup>F-FDG PET/CT).

### *Immunohistochemistry*

Five micromolar FFPE sections were cut, dewaxed, rehydrated, and subjected to antigen retrieval. After blocking endogenous peroxidase activity, the sections were incubated with the primary antibody against PGP9.5 (code Z5116, Dako, CA) (1:200) (overnight at 4°C). Immunohistochemistry was performed using the streptavidin-biotin peroxidase complex method (Lab Vision, Fremont, CA). The slides were examined and pictures were taken using an Olympus BX60 (Olympus, Japan). Sections known to stain positively were incubated in each batch and negative controls were also prepared by replacing the primary antibody with preimmune sera. Positive-stained cells with nuclei were counted and results were expressed as numerical densities. Positive-stained nerves were quantitated by ImageJ software and are expressed as positive area per total counted area.

### *Data collection and follow-up*

The clinical, laboratory, and radiologic records of all patients were retrospectively reviewed (**Table 1**). Liver function tests were checked in all patients every three months in order to evaluate hepatic functional reserve. The results of all 13 HCC patients with synchronous or metachronous bone metastasis were analyzed. Follow-up cross-sectional imaging (contrast-enhanced CT or MRI) was performed one month after treatment. Further treatments were based on clinical evaluation, laboratory values and imaging response. Patients were followed-up every 3 months. The patients were followed up until death or until the date of last follow-up. Follow-up was finished on February 28, 2015. Overall survival was calculated from the date after hepatic resection to the date of death for any cause or last follow-up.

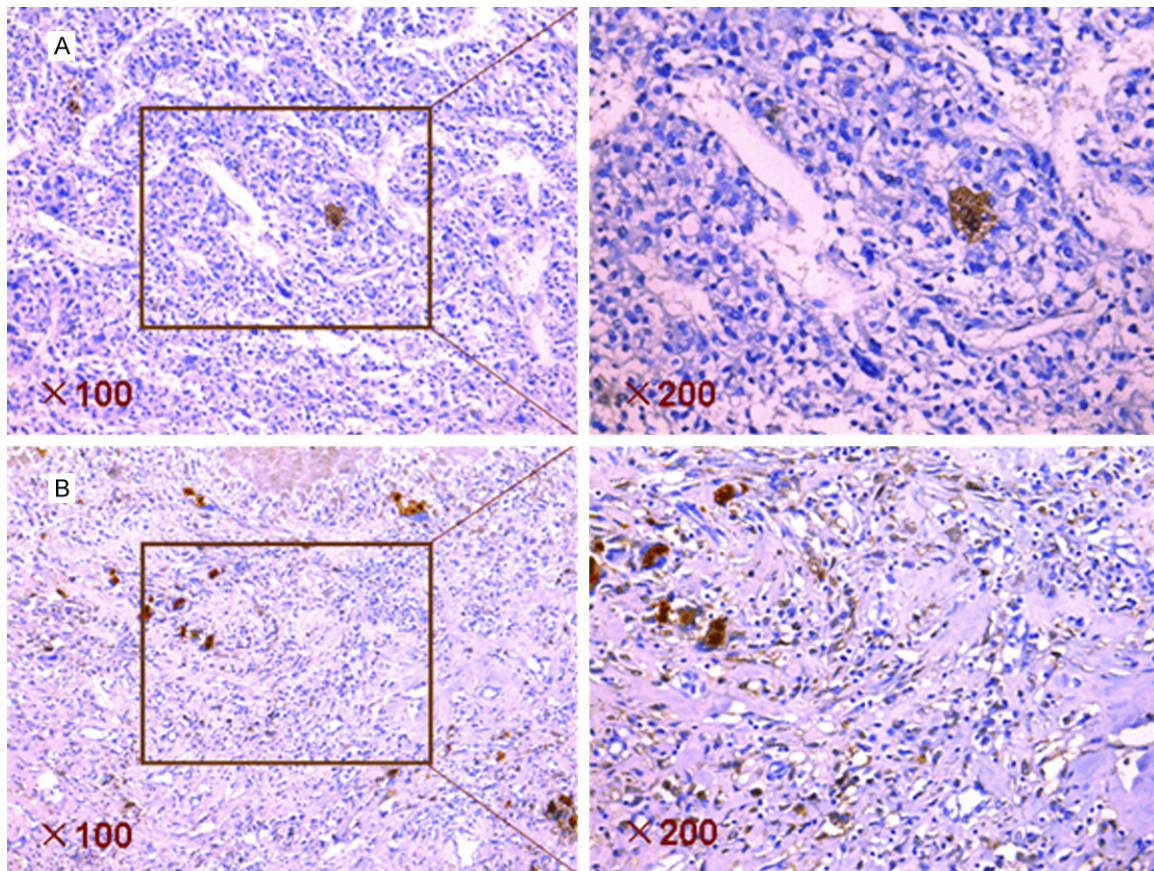
## Neuronal density in HCC bone metastasis

**Table 1.** Clinical characteristics of 13 hepatocellular carcinoma patients with synchronous or metachronous bone metastasis received surgery

	Gender (Male/Female)	Age (Years)	AFP level (ng/ml)	ALT (IU/L)	AST (IU/L)	HBV (Po/Ne)	HCV (Po/Ne)	BCLC staging (A/B/C)	Child's score (A/B/C)	HCC NM	BM (S/M)	OS	Chem (Y/N)	Rad (Y/N)	MTT (Y/N)
Case 1	Female	67	NA	NA	NA	Po	Ne	NA	NA	2	M	84.0	Y	N	N
Case2	Male	39	8.4	NA	NA	Po	Ne	NA	NA	2	M	57.5	Y	Y	Y
Case 3	Male	49	24.2	349	229	Po	Ne	C	B	>3	M	NA	Y	N	N
Case 4	Male	58	27.1	NA	NA	Po	Ne	A	A	2	M	42.3	NA	N	N
Case 5	Male	74	5.7	41	32	Po	Ne	C	A	1	M	NA	Y	N	N
Case 6	Male	43	2335.3	48	83	Po	Ne	B	A	>3	M	3.0	Y	Y	Y
Case 7	Male	62	8.3	33	34	Po	Ne	A	A	1	M	NA	Y	N	N
Case 8	Male	50	14.7	27	117	Po	Ne	C	A	>3	M	25.0	Y	N	Y
Case 9	Male	67	89.6	NA	NA	Po	Ne	A	NA	>3	M	NA	Y	N	N
Case 10	Male	55	4.4	28	30	Po	Ne	NA	A	>3	S	9.3	Y	N	N
Case 11	Male	52	27.5	28	21	Po	Ne	B	A	>3	M	36.0	Y	N	N
Case 12	Male	26	2175.2	16	21	Po	Ne	C	A	3	M	21.6	Y	N	N
Case 13	Male	42	228.9	23	21	Po	Ne	B	A	>3	M	27.5	Y	N	Y

Abbreviations: HBV, hepatitis B virus infection. HCV, hepatitis C virus infection. Po, positive. Ne, negative. AST, aspartate aminotransferase. ALT, aspartate aminotransferase. AFP, alpha-fetoprotein. BCLC staging, Barcelona clinic liver cancer staging. NA, not available. OS, overall survival (months), Chem, chemotherapy. MTT, Molecularly targeted therapy. Rad, radiotherapy. HCC NM, hepatocellular carcinoma number of masses. BM (S/M), bone metastases (S, synchronous; M, metachronous). Y, yes. N, No.





**Figure 1.** Representative immunohistochemical microphotographs showing PGP9.5 in hepatocellular carcinoma (A) and paired bone metastases (B) (original magnification, left column,  $\times 100$ ; right column,  $\times 200$ ).

#### Statistical analysis

Descriptive statistics (means  $\pm$  SED) were provided when appropriate. All statistical analyses were performed with software (SPSS 16.0 statistical package; SPSS, Chicago, Ill). Nerve densities in HCC and paired bone metastases were analyzed via Student's *t* test.  $P < 0.05$  was considered indicative of a statistically significant difference.

#### Results

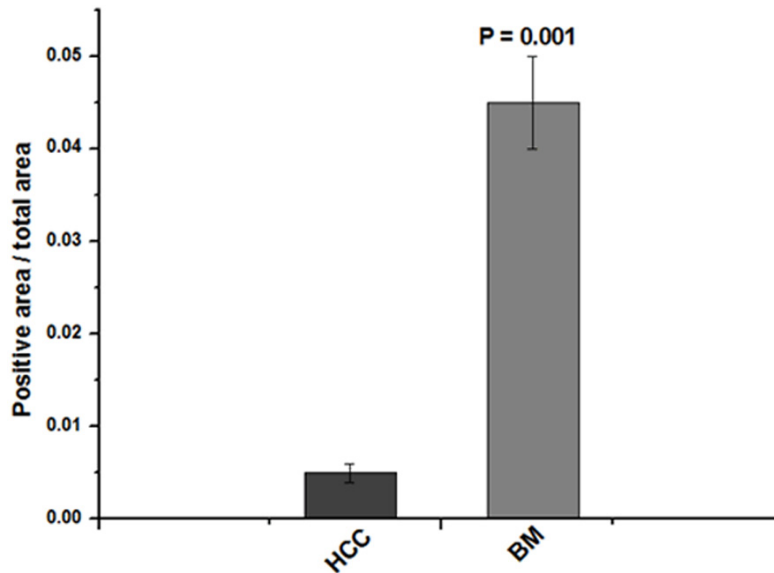
##### *Clinicopathological characteristics of HCC patients*

The clinicopathological characteristics of the cohort are summarized in **Table 1**. The 13 patients (12 males, 1 female) had an age range from 26 to 74 years. Hepatitis B virus (HBV) infection was the most common etiological factor of HCC, seen in 13 (100%) patients. No patient with hepatitis C virus (HCV) infection was found. BCLC B patients were 3 (23.1%),

and BCLC C patients were 4 (30.8%). Child's score A patients were 9 (69.2%), and Child's score B patients were 1 (7.7%). All the patients received synchronous or metachronous resections of the HCC and bone metastases. Of the patients, only 1 patient (7.7%) was treated with surgery alone, 12 (92.3%) patients received adjuvant chemotherapy, 2 (15.4%) patients received adjuvant radiotherapy, and 4 (30.8%) patients received molecularly targeted therapy preoperatively or postoperatively.

##### *Higher nerve density in bone metastases tissues compared with HCC tissues*

PGP9.5 is an ubiquitin-protein hydrolase that is expressed in the neuronal cell bodies and axons in the central and peripheral nervous system. Immunohistochemical analysis of PGP 9.5 in the HCC and bone metastases tissues revealed a higher nerve density in bone metastases tissues compared with HCC tissues (**Figures 1 and 2**).



**Figure 2.** PGP9.5-immunoreactive nerve densities in hepatocellular carcinoma (HCC) and paired bone metastases (BM). Means  $\pm$  SEM (n=13). *P* values were calculated by Student's *t* test.

## Discussion

HCC is the fifth most common cancer in men worldwide [4]. The bone is well known to be the third most frequent site of metastases by all tumors, after the lungs and lymph nodes, and HCC bone colonization has been reported in approximately 20% of patients affected by this tumor [5, 25, 26]. Recently, the progress in both diagnostic modalities and therapeutic procedures, such as surgical resection, radiofrequency ablation, and transcatheter arterial chemoembolization in association with treatments using small molecules as multikinase inhibitors, has prolonged the survival in HCC patients which led to a concurrent worsening of the tumor progression within the skeleton and the formation of bone metastases [5-7, 25]. To date, few data are yet available about bone involvement in patients with HCC, and the nature and the characteristics of bone metastases in HCC have not been fully explored.

A key feature of malignant cells is their ability to dissociate from the primary tumor and to establish metastatic deposits at distant sites. It has long been known that, in addition to being found in vascular and lymphatic systems, cancer cells occur in neuronal spaces, which serve as an alternative route for dissemination. This neuro-invasive ability of cancer cells was first

described as the lateral growth of cancer cells along nerve fibers surrounding the cancer [15, 27, 28]. Why cancer cells invade nerves is as yet not fully known, but it has been postulated that once cancer cells have invaded the outer layers of the neuronal sheath, they become part of an elite and favorable environment [15, 29]. PNI has been broadly defined as tumor cell invasion, in, around and through nerves [30]. It also has been called neurotropic carcinomatous spread and perineural spread. PNI was reported first in the European literature by scientists who described head and neck cancers that exhibited a predilection for growth

along nerves as they made their way toward the intracranial fossa [15]. PNI has emerged since then as a key pathologic feature of many other malignancies, including those of the pancreas, colon and rectum, prostate, biliary tract, and stomach. For many of these malignancies, PNI is a marker of poor outcome and a harbinger of decreased survival [31-35].

In this study, the nerve density was evaluated by immunohistochemistry in FFPE HCC and paired bone metastases tissues from 13 HCC patients with synchronous or metachronous bone metastases that underwent surgical resection. Our results found that HCC bone metastases tissues express higher perineural density than HCC tissues, which indicated a potential association between PNI and bone metastases from HCC.

Several lines of evidence have linked the nervous system to tumor growth and progression. Migration of tumor cells along nerves, a process termed PNI, correlates with poor prognosis in certain epithelial cancers, including prostate cancer and gastric cancer [36-39]. A retrospective blinded analysis of prostate adenocarcinoma specimens from 43 patients revealed that the densities of sympathetic and parasympathetic nerve fibers in tumor and surrounding normal tissue, respectively, were associated

with poor clinical outcomes [40]. Magnon et al. investigated the role that the autonomic nervous system plays in the development and spread of prostate cancer in both mice and human models. The study shows different roles for both branches of the autonomic nervous system, with the sympathetic system promoting early stages of tumorigenesis, and the parasympathetic system promoting cancer dissemination. This information could lead to important new foundations for treatment, therapies and management of prostate cancer [40]. Zhao and colleagues' work suggest that vagal innervation contributes to gastric tumorigenesis [39]. Surgical denervation via vagotomy during the preneoplastic stage of tumorigenesis diminished tumor incidence and size and attenuated tumor cell proliferation specifically in the denervated portion of the stomach, suggesting that the vagus nerve promotes gastric cancer growth [39]. These findings identify nerves as important regulators of gastric stem cell expansion and tumor progression [39]. Their work indicated that denervation might represent a feasible strategy for the control of gastric cancer.

In conclusion, a higher perineural density was found in HCC bone metastases tissues compared with HCC tissues. Our result indicated a potential role of the PNI in bone metastases from HCC. This possible association between the distribution of PNI and the HCC bone metastasis prompted us to study the role of nerves in HCC bone metastasis in future work.

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## Disclosure of conflict of interest

None.

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