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# A clinicopathological model to predict bone metastasis in hepatocellular carcinoma

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**Abstract**
*Background* We aimed to develop a clinicopathological model that would predict the risk of bone metastasis (BM) in hepatocellular carcinoma (HCC).
*Methods* We first evaluated a training cohort of 201 HCC patients who had undergone hepatectomy and found that the following factors independently predicted BM development: vascular invasion, tumor-node-metastasis stage, CXCR4, connective tissue growth factor, and interleukin-11. These variables were used to construct a clinicopathological prediction model that may be scored from 0 to 19. The predictive value of the model was demonstrated in a validation cohort of 179 post-hepatectomy HCC patients.
*Results* During a median follow-up of 54.3 months for the training cohort and 52.5 months for the validation cohort, 23 patients (11.4%) in the former and 19 patients (10.6%) in the latter developed BM. A cutoff value of 9.4 best discriminated BM risk and was able to exclude future BM development with high accuracy in the validation cohort. The sensitivity and specificity of the method were 73.7 and 78.7%, respectively, the positive predictive value was 29.2%, and the negative predictive value 96.2%. The 1- and 2-year cumulative BM rates were (respectively) 10.8% and 27.4% in the high-risk group and 2.4 and 4.3% in the low-risk group. The hazard ratio for BM of the high- versus low-risk group was 9.240 (95% CI: 3.319–25.722).
*Conclusion* The simple prediction model constructed from clinicopathological parameters is accurate in predicting BM development in HCC patients.

**Keywords** Hepatocellular carcinoma · Bone metastasis · Metastasis prediction model · Tissue microarray · Prognosis

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## Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer death in China and the third in the world (Tang 2001; Parkin et al. 2005). Surgical resection plays a major role in treatment for HCC and offers a chance of cure for patients (Bruix et al. 2006; Bruix and Sherman 2005). However, the prognosis for HCC patients with extrahepatic metastasis is poor (Uchino et al. 2011). Bone metastasis (BM) is one of the major sites of extrahepatic metastasis. We have previously reported that the frequency of BM in HCC patients who had undergone curative resection was 11.7% (Xiang et al. 2011a). BM from HCC itself rarely causes death, but it is a cause of pain and other significant symptoms that are detrimental to the quality of the patient's life (He et al. 2009).

We have used a cDNA-mediated annealing, selection, extension, and ligation assay method to screen for predictive BM biomarkers, and we identified intratumoral connective tissue growth factor (CTGF) and interleukin (IL)-11(Xiang et al. 2011a). We also have reported previously that CXCR4 overexpression in primary tumor tissues was associated with BM from HCC (Xiang et al. 2009a).

We therefore have become interested in the possibility of combining these biomarkers with certain clinical factors to establish a model for predicting BM from HCC. Until now, there has been no such prediction model or clinical scoring system. If HCC patients at high risk of BM may be screened out early, there are therapeutic measures (including administration of bisphosphonates) that may be taken to decrease the probability of BM and increase the quality of life.

The aim of the present study was to use the biomarkers and clinical factors, which we identified with relevance to BM, to set up a model to predict BM in HCC patients.

## Methods

### Patients and tissue specimens

The present retrospective study was based on two cohorts. Cohort 1 was used as the training set to derive a model for predicting BM in HCC patients, and cohort 2 served to validate the model.

From May 1999 to October 2005, there were 201 patients with pathologically proven HCC who underwent hepatectomy (all performed by the same surgical team) at the Liver Cancer Institute, Fudan University, and these were included in cohort 1. This group consisted of 171 men and 30 women with a mean age of 50.9 ± 10.3 years (range, 25–81 years). Of these patients, 156 (77.6%) were hepatitis B surface antigen (HBsAg)-positive.

The validation cohort, enrolled from January 2000 to March 2005, comprised 179 pathologically proven HCC patients who had all undergone hepatectomy by a second team at the same institution as cohort 1. This study population consisted of 156 men and 23 women with a mean age of 51.2 ± 10.0 years (range, 25–75 years). Of the 179 patients, 142 (79.3%) were infected with hepatitis B virus.

Tumor size of patients was based on the largest dimension of the tumor specimen. Vascular invasion was determined by microscopic examination of the resected specimen. Tumor stage was determined according to the UICC tumor-node-metastasis (TNM) classification system (6th edition). Tumor differentiation was graded by the Edmondson grading system. All patients received a bone scan prior to surgery to exclude BM. BM was not detected in any of the patients of cohort 1 or 2 at the time of surgery.

The inclusion criteria of tissue specimens were limited to the followings: a curative resection; an HCC diagnosis based on pathology; suitable formalin-fixed, paraffin-embedded tissue; no prior anticancer treatment; and complete clinicopathological and follow-up data for the patient.

The study protocol was approved by the Zhongshan Hospital research Ethics Committee. Informed consent was obtained from each patient in accordance with this committee's regulations.

### Follow-up and postoperative treatment

Follow-up was performed at 3-month intervals after hepatectomy. At every visit, the patient history was taken and a physical examination was performed. Chest radiography was performed every 6 months. Ultrasound images of the liver and abdominal lymph nodes and laboratory tests (liver function, α-fetoprotein, and hematologic parameters) were independently evaluated every 3 months by doctors who had no knowledge of the study. A bone scan was performed annually, and bone scanning or magnetic resonance imaging was immediately performed upon any report of localized bone pain. A diagnosis of BM was based on a history of HCC, 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 as follows: 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.

### Tissue microarray and immunohistochemistry

Tissue microarrays (TMAs) were constructed as previously described (Xiang et al. 2011b, c). Hematoxylin- and eosin-stained TMA slides were screened to identify the optimal intratumoral tissue to use for analysis. TMA slides of training cohort and validation cohort, containing samples from a total of 380 HCC patients, were then constructed in collaboration with Shanghai Biochip Company, Ltd., Shanghai, China. Two tissue cores were collected from non-necrotic areas of tumor foci. Punch cores with a longest dimension of 1.0 mm were used. Sections (4-μm thickness) of the resulting TMA blocks were prepared by using standard techniques.

Immunohistochemistry was carried out as previously described (Xiang et al. 2009b). Primary antibodies used were mouse anti-human CXCR4 monoclonal antibody (R & D Systems, Minneapolis, MN); mouse anti-human CTGF monoclonal antibody, and rabbit anti-human IL-11 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). TMA slides were incubated with primary antibodies overnight at 4°C and then washed to remove excess primary antibody. The Envision-plus system (En-Vision+/HRP/Mo, Dako, Carpinteria, CA) was used for the detection step. 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.

Positive staining was quantified with a computerized imaging system consisting of a Leica CCD camera DFC420 connected to a Leica DM IRE2 microscope (Leica Microsystems Imaging Solutions, Ltd., Cambridge, UK). Immunohistochemical staining was assessed by three of the authors, who were blinded with respect to outcome. For CXCR4, the intensity of staining (brown color) was scored semi-quantitatively, as follows: +, weak; ++, medium; +++, strong; and ++++, very strong. Samples receiving a score of ++ or greater were considered CXCR4-positive (Shim et al. 2006). For CTGF and IL-11, we randomly selected 10 high-power fields (×400magnification; 100 cells/field) and counted 1,000 cells in each core. The percentages of positive cells expressing CTGF and IL-11 were categorized as follows: CTGF was considered highly expressed if there were ≥50% positive cells (Lin et al. 2005); IL-11 expression was considered positive if ≥10% of the cells were reactive (Yamazumi et al. 2006).

### Statistical analysis

Statistical analyses were performed with SPSS 16.0 software (SPSS, Chicago, IL). A simple risk score was devised by using significant variables ($P < 0.05$) obtained from stepwise multivariate analysis. The score was the weighted sum of the variables; the individual variable weights were defined as the quotient (rounded to nearest integer) of the corresponding estimated coefficient from a Cox regression analysis that was then divided by the smallest chi-square ($\chi^2$) coefficient (Wong et al. 2010). The performance of the cutoff was determined by linear trend $\chi^2$ test in terms of the discriminatory ability and monotonicity (Feinstein 1972; Ueno et al. 2001). The score was then categorized as either low- or high-risk group. Time-to-BM was defined as the interval from the date of surgery to the date of presentation of BM, and it was analyzed by Kaplan–Meier and log-rank tests. Area under the curve (AUC) and the 95% confidence interval were used to assess the power of the model for predicting BM. A $P$ value of <0.05 by a two-tailed test was judged to be significant.

## Results

### Patients' background data

The characteristics of all 380 HCC patients (201 in cohort 1 and 179 in cohort 2) are summarized in Table 1. There was no significant difference in incidence of BM between the two cohorts. All patients of the training cohort were observed until January 2010, and the median follow-up time was 54.3 months (range, 3.9–118.9 months). For the validation cohort, observation continued until May 2010 and the median follow-up time was 52.5 months (range, 3.0–116.1 months). During the follow-up time, 23 patients (11.4%) in the training cohort and 19 patients (10.6%) in the validation cohort developed BM.

### Expression of immunohistochemical biomarkers in TMA

CXCR4 staining was detected both in the cytoplasm and in nucleus. CTGF and IL-11 expression was mainly localized in the cytoplasm of tumor cells or hepatocytes. In the training cohort, CXCR4-positive expression was detected in 92 of 201 patients (45.8%), high CTGF expression in 35 (17.4%), and positive IL-11 expression in 51 (25.4%). In the validation cohort, CXCR4 positive expression was detected in 76 of 179 patients (42.5%), high CTGF expression in 34 (19.0%) and positive IL-11 expression in 43 (24.0%).

### Predictors of BM

For the training cohort, 17 clinicopathological features were considered in the Cox proportional hazards regression univariate analysis. These consisted of age, gender, HBsAg, hepatitis C virus antibody, α-fetoprotein, alanine aminotransferase, liver cirrhosis, Child-Pugh score, tumor differentiation, tumor size, tumor number, tumor encapsulation, vascular invasion, TNM stage, CXCR4, CTGF, and IL-11. Table 2 summarizes the association of clinicopathological factors with BM in HCC patients of the training cohort. By univariate comparison, tumor differentiation, tumor number, vascular invasion, TNM stage, CXCR4, CTGF, and IL-11 were significantly associated (all $P < 0.03$) with subsequent BM in HCC patients. Age ($P = 0.859$), gender ($P = 0.486$), HBsAg ($P = 0.118$), HCV-Ab ($P = 0.327$), AFP ($P = 0.332$), ALT ($P = 0.750$), liver cirrhosis ($P = 0.240$), Child-Push score ($P = 0.819$), tumor size ($P = 0.661$), and tumor encapsulation ($P = 0.864$) were not significantly associated with BM. Variables that displayed prognostic significance by univariate analysis were adopted for multivariate analysis. By multivariate analysis, the following five independent variables were found to be significant in predicting the risk of BM: vascular invasion ($P = 0.006$), TNM stage ($P = 0.025$), CXCR4 ($P = 0.005$), CTGF ($P = 0.003$), and IL-11 ($P = 0.001$).

### Derivation of prediction model of BM

In Table 2, the smallest $\chi^2$ of multivariate analysis was 3.157, and then the $\chi^2$ of the five factors (vascular invasion, TNM stage, CXCR4, CTGF, and IL-11) were divided by 3.157. As shown in Table 3, a simple risk score was

**Table 1** Baseline demographics and clinicopathological characteristics of the study population

| Variable | Training cohort | | | | Validation cohort | | | |
|---|---|---|---|---|---|---|---|---|
| | All ($n = 201$) | NBM ($n = 178$) | BM ($n = 23$) | $P$ | All ($n = 179$) | NBM ($n = 160$) | BM ($n = 19$) | $P$ |
| Age, yr | | | | | | | | |
| ≤51 | 104 | 92 | 12 | 0.965 | 89 | 79 | 10 | 0.788 |
| >51 | 97 | 86 | 11 | | 90 | 81 | 9 | |
| Gender | | | | | | | | |
| Female | 30 | 26 | 4 | 0.724 | 23 | 20 | 3 | 0.716 |
| Male | 171 | 152 | 19 | | 156 | 140 | 16 | |
| HBsAg | | | | | | | | |
| Negative | 47 | 42 | 5 | 0.843 | 37 | 35 | 2 | 0.371 |
| Positive | 154 | 136 | 18 | | 142 | 125 | 17 | |
| HCV-Ab | | | | | | | | |
| Negative | 197 | 175 | 22 | 0.387 | 176 | 157 | 19 | 1.000 |
| Positive | 4 | 3 | 1 | | 3 | 3 | 0 | |
| AFP, ng/mL | | | | | | | | |
| ≤20 | 62 | 53 | 9 | 0.361 | 64 | 55 | 9 | 0.264 |
| >20 | 139 | 125 | 14 | | 115 | 105 | 10 | |
| ALT, U/L | | | | | | | | |
| ≤40 | 126 | 113 | 13 | 0.516 | 108 | 98 | 10 | 0.468 |
| >40 | 75 | 65 | 10 | | 71 | 62 | 9 | |
| Liver cirrhosis | | | | | | | | |
| No | 28 | 24 | 4 | 0.610 | 22 | 19 | 3 | 0.709 |
| Yes | 173 | 154 | 19 | | 157 | 141 | 16 | |
| Child-Pugh score | | | | | | | | |
| A | 199 | 176 | 23 | 1.000 | 178 | 159 | 19 | 1.000 |
| B | 2 | 2 | 0 | | 1 | 1 | 0 | |
| Tumor differentiation | | | | | | | | |
| I–II | 146 | 136 | 10 | 0.001 | 122 | 113 | 9 | 0.040 |
| III–IV | 55 | 42 | 13 | | 57 | 47 | 10 | |
| Tumor size, cm | | | | | | | | |
| ≤5 | 103 | 92 | 11 | 0.727 | 89 | 82 | 7 | 0.235 |
| >5 | 98 | 86 | 12 | | 90 | 78 | 12 | |
| Tumor number | | | | | | | | |
| Single | 153 | 141 | 12 | 0.004 | 131 | 121 | 10 | 0.032 |
| Multiple | 48 | 37 | 11 | | 48 | 39 | 9 | |
| Tumor encapsulation | | | | | | | | |
| Complete | 110 | 98 | 12 | 0.794 | 93 | 86 | 7 | 0.163 |
| None | 91 | 80 | 11 | | 86 | 74 | 12 | |
| Vascular invasion | | | | | | | | |
| No | 154 | 144 | 10 | <0.001 | 139 | 129 | 10 | 0.006 |
| Yes | 47 | 34 | 13 | | 40 | 31 | 9 | |
| TNM stage | | | | | | | | |
| I | 143 | 131 | 12 | 0.033 | 125 | 116 | 9 | 0.024 |
| II–III | 58 | 47 | 11 | | 54 | 44 | 10 | |
| CXCR4 | | | | | | | | |
| Negative | 109 | 104 | 5 | 0.001 | 103 | 97 | 6 | 0.015 |
| Positive | 92 | 74 | 18 | | 76 | 63 | 13 | |
| CTGF | | | | | | | | |
| Low | 166 | 151 | 15 | 0.020 | 145 | 134 | 11 | 0.007 |

**Table 1** continued

| Variable | Training cohort | | | | Validation cohort | | | |
|---|---|---|---|---|---|---|---|---|
| | All ($n = 201$) | NBM ($n = 178$) | BM ($n = 23$) | *P* | All ($n = 179$) | NBM ($n = 160$) | BM ($n = 19$) | *P* |
| High | 35 | 27 | 8 | | 34 | 26 | 8 | |
| IL-11 | | | | | | | | |
| Negative | 150 | 137 | 13 | 0.034 | 136 | 126 | 10 | 0.012 |
| Positive | 51 | 41 | 10 | | 43 | 34 | 9 | |

*HBsAg* hepatitis B surface antigen, *HCV-Ab* hepatitis C virus antibody, *AFP* α-fetoprotein, *ALT* alanine aminotransferase, *TNM* tumor-node-metastasis, *CTGF* connective tissue growth factor, *IL* interleukin

**Table 2** Clinicopathological factors associated with bone metastasis of hepatocellular carcinoma in the training cohort

| Clinicopathological factors | Univariate | | | Multivariate | | | |
|---|---|---|---|---|---|---|---|
| | Hazard ratio | 95% CI | *P* | $\chi^2$ score | Hazard ratio | 95% CI | *P* |
| Tumor differentiation | | | | | | | |
| I–II | 1 | | | | | | NS |
| III–IV | 3.436 | 1.506–7.843 | 0.003 | | | | |
| Tumor number | | | | | | | |
| Single | 1 | | | | | | NS |
| Multiple | 3.051 | 1.337–6.961 | 0.008 | | | | |
| Vascular invasion | | | | | | | |
| No | 1 | | | | 1 | | |
| Yes | 5.370 | 2.345–12.294 | <0.001 | 19.815 | 3.278 | 1.395–7.705 | 0.006 |
| TNM stage | | | | | | | |
| I | 1 | | | | 1 | | |
| II–III | 2.497 | 1.100–5.668 | 0.029 | 3.157 | 2.664 | 1.128–6.288 | 0.025 |
| CXCR4 | | | | | | | |
| Negative | 1 | | | | 1 | | |
| Positive | 3.198 | 1.316–7.774 | 0.010 | 7.354 | 3.706 | 1.499–9.161 | 0.005 |
| CTGF | | | | | | | |
| Low | 1 | | | | 1 | | |
| High | 3.589 | 1.552–8.302 | 0.002 | 14.334 | 3.736 | 1.589–8.785 | 0.003 |
| IL-11 | | | | | | | |
| Negative | 1 | | | | 1 | | |
| Positive | 3.799 | 1.674–8.619 | 0.001 | 15.477 | 4.092 | 1.760–9.511 | 0.001 |

*CI* confidence interval, *TNM* tumor-node-metastasis, *CTGF* connective tissue growth factor, *IL* interleukin, *NS* not significant

devised using significant variables in the multivariate model. A score was attributed to each variable according to its relative contribution in the Cox proportional hazards model, as determined by the $\chi^2$ score. Then, every patient was scored according to the five clinicopathological factors status. Every patient got a total score from the sum of the five factors. The scores ranged from 0 to 19 both in the training cohort and in the validation cohort. In the training cohort, the cutoff point of 9.4 was the best in terms of discriminating between low and high risk of BM using linear trend $\chi^2$ test in terms of the discriminatory ability and monotonicity. In the training cohort, using 9.4 as cutoff points, 155/201 patients (77.1%) were in the low-risk category, and 46/201 (22.9%) were high risk. In the low-risk group, 8/155 patients (5.2%) developed BM, and 15/46 (32.6%) developed BM in the high-risk group. In the training cohort, analysis by receiver operating characteristic curve demonstrated that this model can predict BM in HCC patients, with an AUC of 0.809 (95% CI, 0.694–0.923; $P < 0.001$). The prediction sensitivity and specificity were 69.6% and 79.0%, respectively, over 5 years. Cox regression analysis identified that the hazard ratio for BM of the high- versus low-risk groups was 12.132 (95% CI: 5.248–28.049; $P < 0.001$).

**Table 3** Components of the bone metastasis prediction score

| Factor | Score |
|---|---|
| Vascular invasion | |
| No | 0 |
| Yes | 6.3 |
| TNM stage | |
| I | 0 |
| II–III | 1 |
| CXCR4 | |
| Negative | 0 |
| Positive | 2.3 |
| CTGF | |
| Low | 0 |
| High | 4.5 |
| IL-11 | |
| Negative | 0 |
| Positive | 4.9 |

Scores <9.4 indicate low risk and ≥9.4 indicate high risk

*TNM* tumor-node-metastasis, *CTGF* connective tissue growth factor, *IL* interleukin

### Validation of results

In the validation cohort, 19/179 patients (10.6%) developed BM. Forty-eight patients had a score of ≥9.4. Using 9.4 as a cutoff point, 131/179 patients (73.2%) were categorized as low risk, and 48/179 (26.8%) as high risk. In the low-risk group, 5/131 patients (3.8%) developed BM, and 14/48 patients (29.2%) developed BM in the high-risk group. Receiver operating characteristic analysis demonstrated that the present model is able to predict BM in HCC patients (AUC of 0.762; 95% CI, 0.642–0.883; $P < 0.001$). The prediction sensitivity and specificity were 73.7 and 78.7%, respectively, over 5 years. The 5-year positive predictive value was 29.2%, and the negative predictive value was 96.2%.

Time-to-BM was analyzed by Kaplan–Meier and log-rank tests. Patients with a high score (≥9.4) were more likely to develop BM ($P < 0.001$). The 1- and 2-year cumulative BM rates were (respectively) 10.8 and 27.4% in the high-risk group, and 2.4 and 4.3% in the low-risk group. By Cox regression analysis, the hazard ratio for BM of the high- versus low-risk group was 9.240 (95% CI: 3.319–25.722; $P < 0.001$). Log-rank test showed patients in high-risk group had poor disease-free survival ($P = 0.011$) and overall survival ($P = 0.007$) than those in low-risk group.

## Discussion

The increasing incidence of BM that develops from HCC may be attributed to the prolonged survival of HCC patients due to recent progress in both the diagnosis and treatment for the disease (Fukutomi et al. 2001). HCC patients with BM not only have a poor prognosis but also suffer from pain and other significant symptoms that are detrimental to quality of life. Once tumors metastasize to bone, they are usually incurable, the consequences of BM are often devastating (Roodman 2004). Severe pain, pathological fracture, and spinal cord compression may result; additionally, malignant hypercalcemia may develop, which can be life-threatening in patients with BM. We have reported that external beam radiotherapy can effect relief from bone pain (He et al. 2009). But the clinical outcome of HCC patients with BM is still very poor. Only by screening HCC patients at high risk for developing BM, which depends on an effective prediction model, we can carry out more effective individualized therapy that will prevent its development.

In the present study, we have developed a simple model composed of clinicopathological factors to predict the future risk of BM in HCC patients who have undergone curative resection. The predictive model we propose incorporates the tumor properties of vascular invasion and TNM stage, and the expression levels of CXCR4, CTGF, and IL-11 proteins. Of these properties, vascular invasion and TNM stage are known prognostic factors for HCC (Zhu et al. 2008); CXCR4 is known to significantly decrease BM-free survival in vivo, IL-11 to stimulate osteoclasts, and CTGF to enhance BM in vivo (Horak and Steeg 2005; Kang et al. 2003; Xiang et al. 2011a). We have previously reported these factors to be associated with BM in HCC. In this study, we show that they are independent prognostic factors for BM in HCC. The proposed model provides a more refined and systematic stratification of BM risk in HCC, and it has potential clinical implications. The sensitivity and specificity of this model in predicting BM were approximately 70% and 80%, respectively, both in the training and in validation cohorts used in the study. Both cohorts had approximately 25% patients at high risk. Using a score of 9.4 as a cutoff point, the method based on our model may be used to accurately screen HCC patients at high risk of BM. In the validation cohort, the hazard ratio for developing BM of the high-versus low-risk group was 9.240. To the best of our knowledge, this study is the first report of such a prediction model in this disease context. Using this method, HCC patients at high risk of BM may be identified at the time of surgery for additional therapy, for example, by treatment with bisphosphonates, which are known to inhibit BM (Fournier et al. 2010).

The proposed model has some unique features. First, the factors upon which it is based are easily measured in clinical pathology laboratories. Second, it was formulated and then validated in independent cohorts, and the method based on it shows high accuracy in predicting BM. Finally,

this model has the potential to change the way BM is treated during HCC therapy, and it may provide a useful prediction tool for clinicians. The traditional treatment for BM is palliation radiotherapy when symptoms appear; our model adds an earlier screening procedure to identify and treat high-risk patients to prevent the development of BM. Once HCC patients categorized in the high-risk group by this model, more frequent screening of the BM may be required, it help to find BM early. Administration of bisphosphonates may help to decrease the frequency of BM in high-risk group. On the other hand, bone scan may not be necessary for those in the low-risk group after 3 years.

There are some limitations in the present study. As it was a retrospective cohort study, and because of the limited number of patients involved, the results need to be further validated in a prospective study. It was reported that the detection of BM of HCC can be enhanced by PET/CT (Ho et al. 2011), so preoperative PET/CT may be a useful tool to detect BM of HCC.

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**Conflict of interests** None.

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