The involvement of platelets in extra-hepatic metastasis of hepatocellular carcinoma

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Running title: Platelets and extra-hepatic metastasis

Abstract

Aim: Recently, a relationship between platelets and cancer metastasis has been reported. The aim of this study is to elucidate the risk factors for extra-hepatic metastasis (EHM), with emphasis on association with platelets in patients, with hepatocellular carcinoma (HCC).

Methods: We examined risk factors for EHM in 1613 consecutive, newly diagnosed HCC patients by logistic regression analysis (case-control study). We also examined the factors by Cox proportional hazard model in a retrospective cohort fashion in 803 patients who received non-curative treatment for HCC.

Results: In the case-control study, multivariate analysis revealed that high platelet counts [odds ratio (OR)=4.84; 95% confidence interval (CI)=1.29－29.54; p=0.01], high tumor number, and the presence of macroscopic vascular invasion were significantly associated with EHM. In the cohort study, EHM was diagnosed in 71 patients during the study period (mean observation time=23.3 months). On multivariate analysis, high tumor number, high des-gamma-carboxy prothrombin (DCP), and Child-Pugh class A were significantly correlated with EHM, and the patients with high platelet counts tended to develop EHM (OR=1.73; 95% CI=0.99－3.14; p=0.055).

Conclusions: HCC patients with high platelet counts, as well as large numbers of tumors, high serum DCP, and Child-Pugh class A, are at risk for EHM.

Keywords Hepatocellular carcinoma · extra-hepatic metastasis · prognosis · platelet counts

Introduction

The prognosis of hepatocellular carcinoma (HCC) patients has been improved due to the prevalence of surveillance systems and advances in diagnostic and treatment modalities [1–4]. There are several options for the treatment of HCC at early-to-intermediate stages, such as surgery, radiofrequency ablation (RFA), transcatheter arterial chemoembolization (TACE), and hepatic arterial infusion chemotherapy (HAIC). However, in cases with extra-hepatic metastasis (EHM), the only available evidence-based treatment is molecular targeted therapy (sorafenib), and the prognosis of these patients is still poor [5, 6]. Based on the report of the Liver Cancer Group of Japan, the 5-year survival of HCC patients with EHM (TNM stage IVB) is 16.5%, which is much shorter than the average survival of HCC patients (54.2%) [7]. Therefore, information about risk factors for EHM is important in order to decide the best strategies for treating HCC.

EHM, which is known to be closely related to dedifferentiation, is rarely observed when the primary lesion in the liver is well differentiated HCC. Kanda et al. reported on risk factors for EHM, which included vascular invasion of HCC and elevated tumor markers, and most of the factors were related to tumor characteristics [8]. However, little is known about the relationship of EHM to characteristics of tumor-bearing patients.

Recently, several studies using animal models have demonstrated a relationship between platelets and metastasis of cancer [9–11]. The results indicate that EHM tends to occur when platelet counts are high. There are several putative explanations, and one of them is a direct involvement of platelets: i.e., platelets may assist implantation by forming a clot at the target organ and could induce immune escape by the tumor cells.

Frequently, HCC occurs in patients with chronic liver disease and liver cirrhosis. Platelet counts often decrease with the development of liver disease [12]. As a result, the range of platelet counts in HCC patients is wide, meaning that HCC is a good candidate for examining the relationship between platelets and metastasis in human.

In this study, we have sought to elucidate the role of platelets in metastasis by (1) analyzing characteristics of EHM-positive HCC patients at the time of the tumor discovery (case-control study), and (2) by analyzing risk factors of developing EHM in patients who received non-curative treatment for HCC (retrospective cohort study).

Methods

Patients

Among 1721 consecutive, newly diagnosed HCC patients who were admitted to Okayama University Hospital between January 1991 and August 2012, 1613 patients for whom the necessary data was available were selected for a case-control study of EHM-positive and EHM-negative patients.

For a retrospective cohort study, we selected 803 EHM-negative patients who received non-curative treatment (637 patients treated by TACE, and 97 patients by HAIC). Local ablation therapies had been applied to some of the HCCs in 93 of the patients. Patients who developed EHM within the first 2 months were considered to have already had EHM at initiation of therapy and were excluded from the cohort (n=1). 395 patients had a past-history of curative treatment (182 radio-frequency ablation, 68 percutaneous ethanol injection therapy, 19 microwave coagulation therapy, and 126 hepatectomy).

Informed consent for the use of their clinical information was obtained from all patients in this study. The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki, and it was approved by the ethics committee of our institute.

Diagnosis and follow up

In accordance with the American Association for the Study of Liver Diseases (AASLD) guidelines, HCC was diagnosed radiologically by at least two imaging modalities: hyperattenuation in the arterial phase and hypoattenuation in the portal phase on dynamic computed tomography (CT), and/or magnetic resonance imaging (MRI), and tumor staining on angiography. Fine-needle biopsy using abdominal ultrasonography (US) was performed as necessary in 276 patients for confirming the diagnosis. Vascular invasion was diagnosed macroscopically on the basis of dynamic computed tomography/magnetic resonance imaging, or abdominal ultrasonography

After the treatment, all patients were followed with periodical dynamic CT/MRI, abdominal US, blood test every 1–3 months until EHM occurred.

The diagnosis of EHM was based on imaging results from CT, MRI, US, or bone scintigraphy. These tests were performed when we observed symptoms compatible with EHM, such as pain or neurological impairment, or when HCC-specific tumor markers were elevated. Alpha-fetoprotein (AFP), des-gamma-carboxy prothrombin (DCP), and lectin-reactive AFP (AFP-L3) were used as HCC-specific tumor markers.

Statistical procedures

The association between EHM and 16 clinical parameters was analyzed. Variables included platelet counts, gender, age, viral markers (hepatitis B virus surface antigen and hepatitis C virus antibody), maximum tumor size, number of tumors, vascular invasion, serum tumor markers (AFP and DCP), Child-Pugh class, albumin, total bilirubin, prothrombin time, aspartate aminotransferase (AST), and alanine aminotransferase. We determined the cut-off value of the laboratory data based on median value. In the retrospective cohort study, we used the laboratory data on admission for the initial non-curative treatment (before the treatment). We included a variable "the presence of splenomegaly" in the analysis in addition to the 16 parameters.

Logistic regression analysis was used in the case-control study. Variables that demonstrated a p-value of <0.05 in univariate analysis were entered into the multiple logistic regression model.

Survival and incidence of extra-hepatic metastasis was compared using the Kaplan–Meier method, and the difference was evaluated by log-rank test. Cox's proportional hazard model was used for estimating the risk for EHM in the retrospective cohort study. All statistical analyses were performed using JMP version 9 software (JMP Japan, Tokyo, Japan). All reported p-values are 2-sided, and $p<0.05$ was considered statistically significant.

Results

Case-control study

Patients

At the initial treatment, there were 30 EHM-positive patients and 1583 EHM-negative patients (**Table 1**). The sites of EHM were as follows: lung in 14 patients, bone in 11, lymph node in 10, adrenal gland in 3, and peritoneum in 2. Four patients had EHM in multiple organs. Median survival time (MST) was 3.4 months in EHM-positive patients and 67 months in EHM-negative.

Risk factors for the presence of EHM

Univariate logistic regression analysis revealed that high platelet counts (>$10\times10^4/\mu L$), maximum tumor size (>30 mm), number of tumors (≥4), the presence of vascular invasion, elevated DCP (>40 mAU/ml), elevated AST (>55 IU/L), and the presence of hepatitis C virus antibody were significant risk factors for EHM (**Table 2**). In multivariate analysis of parameters that showed significant differences in univariate analysis, high platelet counts [odds ratio (OR)=4.84; 95% confidence interval (CI)=1.29–29.54; p=0.01], multiple tumors (≥4) (OR=3.01; 95% CI=1.15–8.51; p=0.02), and the presence of vascular invasion (OR=6.94; 95% CI=2.16–26.68; p=<.001) were the risk factors for the presence of EHM.

Retrospective cohort study

Patients and EHM

There were 602 males (75%) in the study, with median age of 69 (range: 23–94) years. Positive HCV antibody was seen in 586 patients (73%), and 125 patients (16%) were HBs antigen-positive. Seventeen patients (2%) were coinfected with HCV and HBV. The median maximum tumor size was 24 mm (range: 5–200mm), and the median number of HCC nodules was 3. There were 162 patients (21%) with tumor vascular invasion. The median platelet counts was $10.6\times10^4/\mu L$ (range: $2.2\times10^4/\mu L$–$65.3\times10^4/\mu L$). Preserved liver function as Child-Pugh class A was seen in 516 patients (67%).

The average observation period was 23.3 months. During observation period, EHM were diagnosed in 71 patients. The sites of newly appeared EHM were as follows: lung in 35 patients (4.4%), bone in 25 (3.1%), lymph node in 12 (1.5%), and adrenal grand in 12 (1.5%). The cumulative incidence of EHM at 0.5, 1, 2, and 5 years was 1.6%, 4.5%, 9.2%, and 22.9%, respectively. The cumulative survival after the diagnosis of EHM was as follows: 59.5% at 6 months, 24.5% at 1 year, 11.2% at 2 years, and 4.5% at 5 years.

Risk factors for EHM

Among the patients who received non-curative treatment, the incidence of EHM at 0.5, 1, and 2 years was 2.0, 6.2, and 13.0%, respectively, in the $>10\times10^4$-platelets group; and it was 0.6, 2.1, and 4.2%, respectively, in the $\leq10\times10^4$-platelets group. A significant difference between these data from the two groups (p=0.002) was observed (**Fig. 1**). No correlation was observed between the site of EHM and platelet counts.

The 16 parameters at the time of the initial non-curative treatment were analyzed to determine the risk factors for the occurrence of EHM by using Cox's proportional hazard model. By univariate analysis, the following parameters were significantly associated with EHM: high platelet counts ($>10\times10^4$ /μL), maximum tumor size (>30mm), number of tumors (≥4), the presence of vascular invasion, HBV infection, HCV infection, elevated DCP, and Child-Pugh class A (**Table 3**). No significant correlation was observed between splenomegaly and EHM. On multivariate analysis for the above 8 parameters exhibiting significance in the univariate analysis, number of tumors (≥4) [hazard ratio (HR)= 3.38; 95% CI=1.94–6.16; p<0.001], elevated DCP (HR=2.67; 95% CI=1.43–5.25; p=0.001), and Child-Pugh class A (HR=2.06; 95% CI=1.07–4.39; p=0.02) were the risk factors for EHM. There was a tendency toward development of EHM in patients with high platelet counts (HR=1.73; 95% CI=0.99–3.14; p=0.055).

Discussion

In this work, we examined the relationship between EHM and clinical parameters, including platelet counts, in two different studies. In the case-control study with newly discovered HCC patients, platelet counts in EHM-positive patients was higher than that in EHM-negative patients. The number of tumors and presence of vascular invasion also correlated with EHM at the time of the first treatment. In the subsequent retrospective cohort study among patients who received non-curative treatment, the risk factors for EHM were identified as elevated serum DCP, multiple tumor nodules, and Child-Pugh class A. There was a tendency for high platelet counts to correlate with EHM, although this was not statistically significant.

We observed a clear relationship between platelet counts and EHM of HCC in the case-control study. However, it was unclear whether high platelet counts promoted EHM or was a consequence of EHM. In cancer patients, the presence of infectious disease and cytokine production by cancer cells may cause an elevation of platelet counts. To eliminate these uncertainties, we performed a retrospective cohort study, and here also we observed that high platelet counts floated as a risk factor for EHM. Platelet count also correlated with the appearance of PVTT in our preliminary analysis, indicating the importance of platelet in various clinical aspects.

The elevation of DCP, the presence of vascular invasion, and multiple nodules of HCC can be considered as risk factors of EHM associated with high malignant potential of HCC. The marker DCP is associated with portal vein invasion and tumor angiogenesis [13], and it also correlates with autologous HCC cell proliferation in vitro through the DCP-Met-STAT3 signaling pathway [14]. Moreover, portal vein invasion is a major cause of intrahepatic metastasis [15]. All of these markers are so-called tumor factors that are characteristic of HCC.

In contrast, high platelet counts is the only risk factor for EHM other than tumor factors. There are several reports that platelets contribute to tumor metastasis [9–11]. P-selectin mediates the aggregation of activated platelets and tumor cells [16], whereby the platelets can then defend the aggregated tumor cells by forming a physical barrier against attack of circulating immune-competent cells [17]. Surviving tumor cells arrest within the microvasculature of distant organs and then subsequently exit from the blood circulation and form metastatic lesions. There are some reports that high platelet counts correlates with poor prognosis in cancers of several organs, including uterus, kidney, brain, pancreas, lung, colon, and breast [18–24]. It is well known that liver function correlates with platelet counts, where platelet counts declines with advancement of liver functional impairment [12]. Addario et al. reported that HCC patients with better hepatic function show an increased risk for metastases [25]. The results of these reports

suggested an indirect relationship between platelet counts and EHM of HCC, and we have confirmed that relationship in the present study. In addition, high platelet counts (>$10\times10^4/\mu L$) was significantly associated with the presence of portal vein tumor thrombosis (PVTT) in both the case-control study ($p<0.001$) and the retrospective cohort study ($p<0.001$). High platelet counts may associated with the appearance of PVTT.

In this study, the frequency of EHM was high in patients with Child- Pugh A. Similar result was reported by Addario et al. as described previously [25]. It is hard to verify the reason; however, we speculated it as follows. It is well known that Child-Pugh class closely correlates with survival of HCC patients. It takes a certain period to metastasize to other organs so that the HCC patients with Child-Pugh B or C might die before the emergence of EHM. As the result, Child-Pugh A arose as a risk factor for EHM.

There are few reports examining the relationship between EHM of HCC and clinical parameters, including platelet counts. Kanda et al. reported that advanced intrahepatic lesions, presence of vascular tumor invasion, elevated tumor markers, and presence of viral hepatitis were risk factors for EHM [8]. However, platelet counts was not selected as the significant risk factor of EHM. The reason for this discrepancy with our results is not clear; however, the difference of timing in the enrollment of patients might be a possible factor. Our

cohort study analyzed the parameters at the first non-curative treatment; whereas, in most existing reports in the literature, the clinical parameters of the patients at the time of the first treatment have been used. However, HCC usually recurs several times, and the clinical course is long. Therefore, the clinical parameters at the time of the first treatment might not directly reflect the characteristics of the patients at the time of EHM development.

There are some limitations in the current study. This experimental design is retrospective and was carried out as a single-center study. The number of patients was relatively small, and we did not observe statistically significant correlations between platelet counts and EHM in the cohort study, although a clear tendency was observed ($p=0.055$). In addition, the mechanism that platelets contribute to EHM of HCC has not been validated in vitro.

From this study, which was carried out in two different experimental settings, we conclude that high platelet counts, large numbers of tumors, elevated DCP, and a good Child-Pugh class are risk factors for EHM in patients with HCC. The results suggest that patients with high platelet counts should be followed up carefully as patients at great risk for EHM.

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varices are risk factors for metastasis of hepatocellular carcinoma. Dig Liver Dis. 2011; 43 (4): 319-24.

Figure legends

Figure 1 Incidence of extra-hepatic metastasis after initial non-curative treatment.

The incidence of extra-hepatic metastasis at 6 months, 1 year, and 5 years were 2.0, 6.2, and 28.4%, respectively, in high platelets group (>10×10^4/μL, solid line); and those in low platelets group (≤10×10^4/μL, dotted line) were 0.6, 2.1, and 16.0%, respectively. A significant difference was observed between the two groups ($p=0.002$).

Table 1. Patient characteristics at first treatment (n=1613)

Variables	EHM-positive (n=30)	EHM-negative (n=1583)
Gender (male)	25 (83%)	1126 (71%)
Age (years)	63 (34－81)	66 (23－94)
Viral markers		
HCV	11 (37%)	1107 (70%)
HBV	7 (23%)	216 (14%)
HCV + HBV	1 (3%)	37 (2%)
Maximum tumor size (mm)	80 (11－170)	23 (8－200)
Number of tumors		
≥4 (including massive and diffuse types)	20 (67%)	256 (17%)
Vascular invasion (present)	23 (77%)	227 (15%)
AFP (ng/ml)	84.4 (1.2－548867)	26.3 (0.6－453265)
DCP (mAU/ml)	3932 (7－410500)	26 (0－1323600)
Platelet counts ($\times 10^4/\mu l$)	21.5 (6－36.5)	10.9 (1.5－242)
Child-Pugh grade (A/B/C)	21/9/0	1028/285/38
Alb (g/dl)	3.77 (2.5－4.7)	3.66 (1.54－5.1)
T.Bil (mg/dl)	0.8 (0.33－4.44)	0.87 (0.16－8.33)
PT (%)	92 (57－134)	91 (10.3－152)
AST (IU/L)	72.5 (14－243)	54 (12－631)
ALT (IU/L)	55.5 (11－135)	47 (4－1082)

Numbers in the tables are median values (ranges) unless otherwise noted.

Abbreviations: EHM, extra-hepatic metastasis; HCV, positive for hepatitis C virus antibody; HBV, positive for hepatitis B virus antigen; AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin; Alb, albumin; T.Bil total bilirubin; PT, prothrombin time; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Table 2. Risk factors of extra-hepatic metastasis at the time of the initial treatment

Variables	Univariate		Multivariate	
	Odds ratio (95% CI)	p value	Odds ratio (95% CI)	p-value
Platelet counts (>10×10⁴/μl)	10.61 (3.17－65.86)	<.001*	4.84 (1.29－29.54)	0.01*
Age (>70 years)	0.49 (0.18－1.14)	0.10		
Gender (male)	2.01 (0.83－5.98)	0.12		
AFP (>10 ng/ml)	1.13 (0.51－2.75)	0.75		
DCP (>40 mAU/ml)	3.87 (1.58－11.60)	0.002*	0.65 (0.21－2.33)	0.48
Maximum tumor size (>30 mm)	8.53 (3.52－25.40)	<.001*	2.53 (0.59－13.52)	0.21
Number of tumors (≥4)	10.02 (4.74－22.56)	<.001*	3.01 (1.15－8.51)	0.02*
Vascular invasion (present)	18.64 (8.30－47.46)	<.001*	6.94 (2.16－26.68)	<.001*
HCV	0.24 (0.11－0.50)	<.001*	0.44 (0.19－1.01)	0.06
HBV	1.85 (0.76－4.06)	0.15		
Child-Pugh grade (A)	0.69 (0.32－1.62)	0.38		
Alb (>3.5 g/dl)	1.24 (0.59－2.73)	0.55		
T. Bil (>1 mg/dl)	0.82 (0.37－2.31)	0.61		
PT (90%)	1.30 (0.63－2.76)	0.47		
AST (>55 IU/L)	2.13 (1.01－4.78)	0.04*	1.56 (0.63－4.13)	0.33
ALT (>50 IU/L)	1.36 (0.66－2.86)	0.39		

95% CI, 95% confidence interval. Other abbreviations are the same as those listed in Table 1.

* Significant value

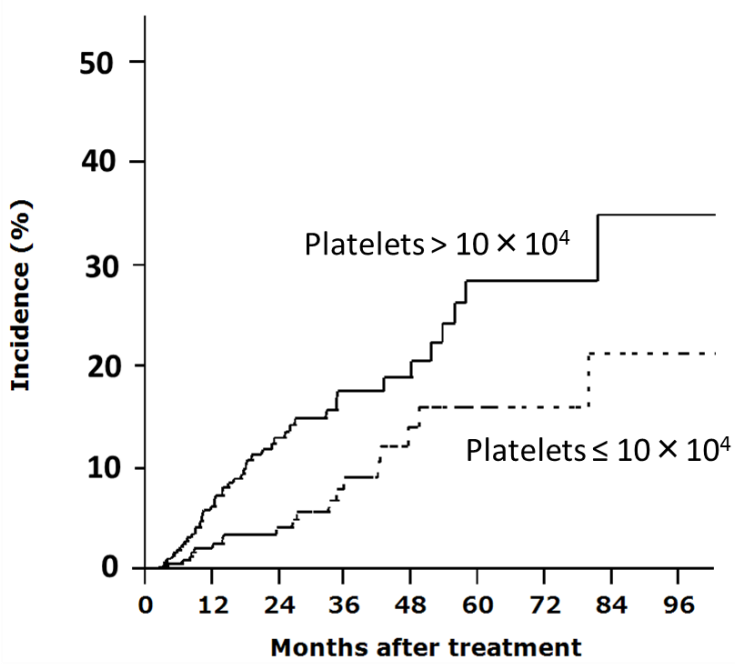
Table 3. Risk factors of extra-hepatic metastasis after non-curative treatment

Variables	Univariate		Multivariate	
	Risk ratio (95% CI)	p-value	Risk ratio (95% CI)	p-value
Platelet counts (>10×10⁴/μl)	2.17 (1.31－3.72)	0.002*	1.73 (0.99－3.14)	0.055
Age (>70 years)	0.73 (0.43－1.21)	0.23		
Gender (male)	1.69 (0.92－3.42)	0.08		
AFP (>10 ng/ml)	1.64 (0.95－3.02)	0.07		
DCP (>40 mAU/ml)	3.49 (1.96－6.69)	<.001*	2.67 (1.43－5.25)	0.001*
Maximum tumor size (>30 mm)	2.31 (1.43－3.76)	<.001*	1.22 (0.66－2.22)	0.5
Number of tumors (≥4)	3.79 (2.29－6.55)	<.001*	3.38 (1.94－6.16)	<.001*
Vascular invasion (present)	2.79 (1.66－4.59)	<.001*	1.27 (0.67－2.41)	0.45
HCV	0.52 (0.32－0.85)	0.01*	0.81 (0.43－1.60)	0.54
HBV	1.75 (1.01－2.92)	0.04*		0.34
Child-Pugh grade (A)	2.16 (1.18－4.36)	0.01*	2.06 (1.07－4.39)	0.02*
Alb (>3.5 g/dl)	1.68 (1.04－2.81)	0.03*		
T. Bil (>1 mg/dl)	0.53 (0.31－0.89)	0.01*		
PT (90 %)	1.37 (0.85－2.25)	0.19		
AST (>55 IU/L)	0.81 (0.49－1.31)	0.39		
ALT (>50 IU/L)	0.81 (0.48－1.32)	0.40		
Splenomegaly	0.84 (0.40－1.89)	0.66		

95% CI, 95% confidence interval. Other abbreviations are the same as those listed in Table 1.

* Significant value

Figure 1. Incidence of extra-hepatic metastasis after initial non-curative treatment.



No. at risk									
Platelets > 10 × 10^4	426	260	146	84	51	27	17	11	5
Platelets ≤ 10 × 10^4	347	221	141	78	47	28	19	15	13