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Platelet activation status in the diagnosis and postoperative prognosis of hepatocellular carcinoma

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ABSTRACT

Background: The venous thromboembolism, which may be caused by increased platelet activation, is a risk factor for tumor prognosis. We determined the platelet activation status for diagnosis and predicting postoperative prognosis of hepatocellular carcinoma.

Methods: We conducted a prospective study of 191 patients diagnosed with HCC at Zhongshan Hospital from April 2016 to July 2016 as well as 99 healthy people. The platelet activation status was assessed by 2 platelet markers, PAC-1 and CD62p, using flow cytometry. The patients were treated with TACE or resection and monitored for ≥6 months. The diagnostic value of marker-positive platelets was determined by the receiver operating characteristic curve and the postoperative value were analyzed using the Kaplan-Meier method and COX regression model.

Results: All the 3 groups with high levels of marker-positive platelets were likely to be diagnosed with HCC and the PAC-1$^+$ percentage had the best efficacy. The univariate analysis showed that the levels of PAC-1$^+$ and CD62p$^+$ platelets was risker factors for poor postoperative prognosis after both TACE and resection. Moreover, the multivariate analysis revealed that the level of PAC-1$^+$ platelets was an independent risk factor for poor prognosis.

Conclusions: The PAC-1$^+$ percentage of platelets is a new indicator for diagnosis and predicting postoperative prognosis.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant tumors worldwide, and its annual morbidity and mortality are constantly increasing [1], which makes it the second leading cause of cancer-related mortality today. For patients with HCC, the most effective treatment is surgical resection. However, not all the patients can undergo surgery due to excessive tumor or distant metastasis. Instead, they are treated with transcatheter arterial chemoembolization (TACE) or radiofrequency ablation. No matter what treatment is received, the patients still have a high risk of postoperative recurrence [2]. Therefore, finding a biomarker that could be used to predict postoperative prognosis has become a mainstream trend in recent HCC researches.

Platelet is one of the important components of peripheral blood. In the exogenous coagulation pathway, factor VII (with tissue factor) acts as the main activator and participates in the entire exogenous coagulation. It has been reported that factor VII is greatly increased in tumor patients, thereby activating thrombin release [3]. The venous thromboembolism has been shown a risk factor for tumor prognosis [4,5]. Especially in the advanced stage of the tumor, abnormal blood emboli have an important influence on the recurrence and metastasis of tumor and the prognosis of the disease [6]. In addition, liver tumor tissue releases more prothrombin than normal liver tissue, leading to activation of platelets [7]. Therefore, the platelet function probably reflects the conditions of HCC patients. Nevertheless, in the early stage of tumorigenesis, thrombosis is often undetectable, or the thrombi are not formed. The formation of blood blots relies on the activation of platelets. The abnormal thrombosis could be caused by abnormal platelet activation, which occurs earlier than the appearance of thrombi. It is possible that, in HCC patients, the abnormal platelet activation recruits

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more platelets, thereby initiating thrombosis and contributing to the poor prognosis. Therefore, we might be able to predict the prognosis of HCC by measuring platelet activation in HCC patients.

To examine the relationship between platelet activation and the development of HCC, we established a flow detection platform to assess platelet activation in peripheral blood. Two markers, PAC-1 (antibody that recognizes the activated GPIIb/IIIa complex) and CD62p (also known as P-selectin), were used to analyze the stages of platelet activation. The GPIIb/IIIa complex is formed and activated on the membrane within a few seconds after platelet activation [8]. The activated complex can be recognized by PAC-1 antibody. In flow cytometry, PAC-1 is used as a marker for early activation of platelets. CD62p, which is mainly present in the alpha particles of platelets, appears on the surface of platelets during platelet activation. It appears later than GPIIb/IIIa complex and is used as a marker of later platelet activation [9].

In the current investigation, we evaluated the role of abnormally activated platelets in the diagnosis of HCC and the postoperative prognosis of metastasis and recurrence. Our results show that patients with HCC or poor prognosis had higher levels of PAC-1- and CD62p-positive platelets, and the PAC-1^+ percentage of platelets had better efficacy. This findings provided functional insights into the connection between abnormally activated platelets and HCC progression and broaden our understanding that platelets could be a promising marker for the diagnosis and prognosis in the HCC patients.

2. Materials and methods

2.1. Enrollment and specimen collection

Ethical approval for the use of human subjects was obtained from the Zhongshan Hospital Research Ethics Committee and informed consent was obtained from each patient. A total of 191 patients with HCC were enrolled. At the same time, 99 healthy controls were enrolled. Inclusion criteria: patients were diagnosed with HCC according to the Code for Diagnosis and Treatment of Primary Liver cancer (2015 edition) and age ≥ 18 y. Exclusion criteria: (1) previous history of other tumors; (2) merger with other malignant tumors; (3) treatment in another hospital before admission. 5 ml of blood anticoagulated with sodium citrate was collected from each HCC patient (before operation) or healthy volunteer. Among these HCC patients, 62 underwent radical resection and 129 received TACE.

2.2. Detection of platelet markers by flow cytometry

Five microliters of anticoagulated blood was mixed gently with anti-CD61-Percp-Cy5.5, anti-CD62p-PE and PAC-1-FITC antibodies (5 ul of each; BD Pharmingen™, USA; completed within 30 min after blood withdrawal). The mixture was incubated in a dark room for 20 min and diluted in 200 μl of HEPES buffer (HEPES buffer configuration: 10 mmol/l HEPES, 137 mmol/l sodium chloride, 2.8 mmol/l potassium chloride, 1 mmol/l magnesium chloride, 12 mmol/l sodium bicarbonate, 0.4 mmol/l sodium dihydrogen phosphate, 5.5 mmol/l glucose) [10]. The diluted samples were analyzed by flow cytometry using FACSCanto II system (BD FACSCanto™). The marker-positive percentage = number of marker-positive platelets/number of total platelets × 100%.

2.3. Judgment of patient prognosis

Postoperative prognosis of TACE treatment was evaluated based on magnetic resonance imaging (MRI) data. Two imaging doctors performed a double-blind review of imaging data.

Complete response (CR): all target lesions disappeared, no new lesions appeared, and tumor markers were normal, maintained for at least 4 weeks. Partial remission (PR): the sum of the maximum diameters of the target lesions was reduced by ≥30% for at least 4 weeks. Progressive disease (PD): the maximum diameter of the target lesion increased by at least ≥20% or new lesions appear. Stable disease (SD): the maximum diameter of the target lesion was reduced to less than PR (partial response), or increased to less than PD. We defined PD and SD as tumor progression, PR and CR as tumor non-progression. Postoperative prognosis of resection was also assessed by 2 imaging doctors using a double-blind examination of imaging MRI data. The presence of recurrence and metastasis was evaluated to determine if the condition was progressive or non-progressive.

2.4. Statistical analysis

Statistical analysis was performed using Medcalc (10.4.7.0) and SPSS 19.0. Student's *t*-test was used to compare the difference between 2 groups. The receiver operating characteristic curve (ROC) was used to evaluate the diagnostic ability of PAC-1^+, $CD62p^+$ (CD62p-positive), PAC-1^+CD62p^+ (PAC-1-positive and CD62p-positive) platelets. Cut-point values were analyzed using X-tile 3.6.1. Kaplan-Meier method was used to generate the survival curves and the log-rank test was used to assess the difference between 2 groups. The COX regression model was used to evaluate independent risk factors. A p value $< .05$ was considered to have statistical significance ($^*p < .05$, $^{**}p < .01$, $^{***}p < .001$).

3. Results

3.1. Application of platelet markers in the diagnosis of hepatocellular carcinoma

One hundred and ninety-one patients were diagnosed with HCC and 99 healthy volunteers were included as controls. There were no differences of age or sex between the 2 groups (Supplemental Table 1). The platelets in the peripheral blood were analyzed by flow cytometry and 2 platelet markers were measured. The $SSC^{low}CD61^+$ platelets were gated based on the PAC-1 and CD62p signals (Fig. 1A). In healthy controls, PAC-1^+ platelets were 13.98 ± 7.95% of total, whereas in HCC patients, the level was 23.82 ± 13.64% (Fig. 1B, left). $CD62p^+$ platelets were 9.50 ± 4.96% of total in healthy people and 15.56 ± 9.33% of total in HCC patients (Fig. 1B, middle). In addition, the percentage of PAC-1^+CD62p^+ platelets was 6.70 ± 4.75% in healthy people and 11.25 ± 8.04% in HCC patients (Fig. 1B, right). All these results indicate that the level of activated platelets increased in the HCC patients.

To determine whether the levels of PAC-1^+, $CD62p^+$ and PAC-1^+CD62p^+ platelets could be potential diagnostic markers for HCC, the ROC curves were generated (Fig. 2). The detailed statistical results were listed in Table 1. The curve of PAC-1^+ percentage (= number of PAC-1^+ platelets / number of total platelets × 100%) had the largest area under the curve among 3 markers (AUC, 0.726; 95% confidence interval [95% CI], 0.670–0.776) and the curve of PAC-1^+CD62p^+ percentage (= number of PAC-1^+CD62p^+ platelets / number of total platelets × 100%) had the smallest AUC (0.661, 95% CI, 0.603–0.715). The AUC for $CD62p^+$ percentage (= number of $CD62p^+$ platelets / number of total platelets × 100%) was 0.697 (95% CI, 0.641–0.750). At a cut-off value (Youden index) of 21.8%, the sensitivity and specificity for PAC-1^+ percentage were 48.68% and 87.88%, respectively. The sensitivity and specificity for $CD62p^+$ percentage (cut-off value, 18.4%) were 39.27% and 95.96%, respectively. In addition, the PAC-1^+CD62p^+ percentage (cut-off value, 12.3%) had 39.27% sensitivity and 88.89% specificity. The results above show that PAC-1 had better diagnostic efficiency for HCC.

In addition, we compared these 3 marker with AFP, which was known as a classic diagnostic marker. The diagnostic efficacy of PAC-1 and AFP (AUC, 0.734; 95% confidence interval [95% CI], 0.679–0.784) was quite similar. Therefore, we further combined these 2 marker to verify its diagnostic value in HCC and found the diagnostic performance

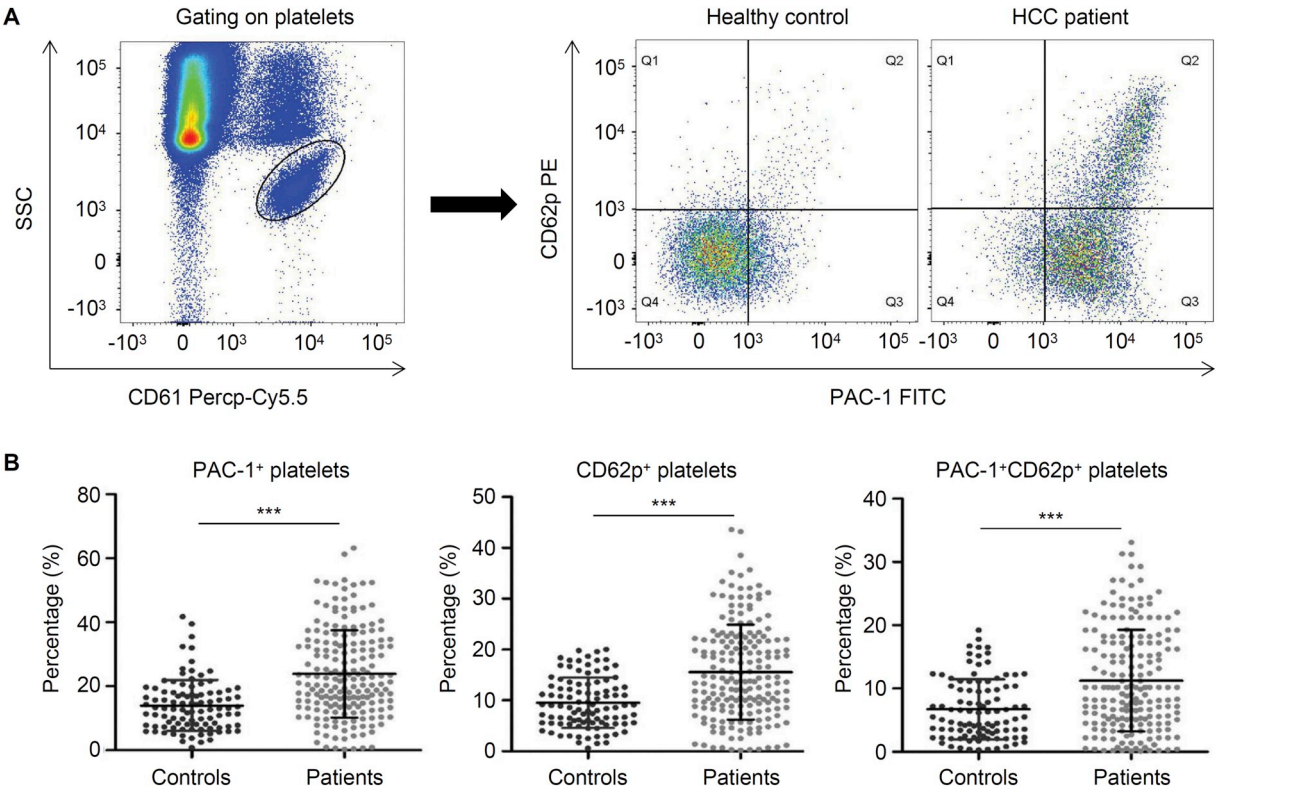

Fig. 1. PAC-1$^+$ and CD62p$^+$ platelets in HCC patients. (A) Detection of PAC-1 and CD62p signals on the platelets in flow cytometry. The platelets were properly defined according to the CD61 expression. The PAC-1 and CD62p signals were analyzed. (B) Percentages of PAC-1$^+$, CD62p$^+$ and PAC-1$^+$CD62p$^+$ platelets in HCC patients (n = 191) and healthy controls (n = 99). ***p < .001.

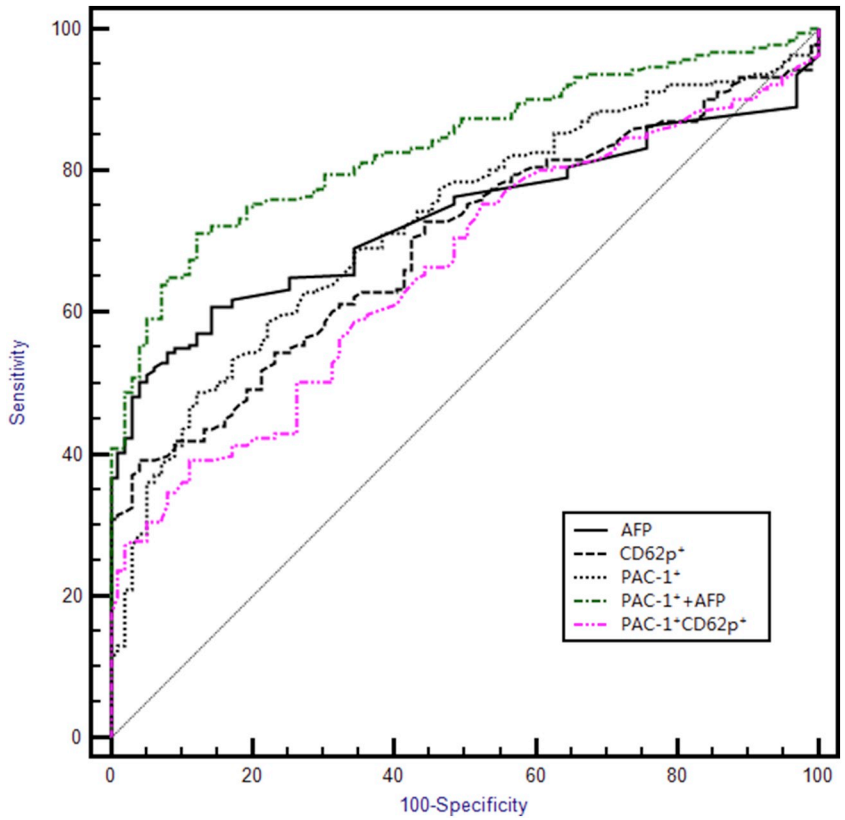

Fig. 2. ROC curves for PAC-1$^+$, CD62p$^+$ or PAC-1$^+$CD62p$^+$, AFP and PAC-1$^+$ + AFP percentage in distinguishing HCC patients from healthy people.

Table 1
Diagnostic efficacy of PAC-1$^+$, CD62p$^+$ or PAC-1$^+$CD62p$^+$, AFP and PAC-1$^+$ + AFP percentage.

	AUC	95% CI	Critical Value (%)	Specificity (%)	Sensitivity (%)
PAC-1$^+$	0.726	0.670–0.776	21.8	87.88	48.69
CD62p$^+$	0.697	0.641–0.750	18.4	95.96	39.27
PAC-1$^+$CD62p$^+$	0.661	0.603–0.715	12.3	88.89	39.27
AFP	0.734	0.679–0.784	10.0	85.86	65.73
PAC-1$^+$ + AFP	0.836	0.788–0.877	0.6	87.88	71.20

AUC, the area under the curve; CI, confidence interval.

Table 2
Clinical information of HCC patients enrolled.

	TACE Group		Reaction Group	
	n = 129	%	n = 62	%
Age				
≤50	35	27.13	24	38.71
> 50	94	72.86	38	61.29
Sex				
Female	17	13.18	8	12.90
Male	112	86.82	54	87.10
AFP				
≤400 ng/ml	56	43.41	38	61.29
> 400 ng/ml	73	56.58	24	38.71
HBsAg				
No	11	8.53	13	20.97
Yes	118	91.47	49	79.03
Liver cirrhosis				
No	9	6.98	16	25.80
Yes	120	93.02	46	74.19
Tumor number				
Single	115	89.15	39	62.90
Multiple	14	10.85	23	37.09
Tumor size				
≤5 cm	16	12.40	25	40.32
> 5 cm	113	87.60	37	59.68
Tumor differentiation				
BCLC 0-A	6	4.65	15	24.19
BCLC B-D	123	95.35	47	75.80
Portal vessel invasion				
No	53	41.08	39	62.90
Yes	76	58.91	23	37.09

BCLC, Barcelona clinic liver cancer staging.

was significantly improved. The AUC for PAC-1$^+$ + AFP was 0.836 (95% CI, 0.788–0.877).

3.2. The treatment for the HCC patients

The physicians evaluated the patients based on their clinical conditions and then offered different operations (Table 2). Among these patients, 129 underwent TACE and 62 underwent radical resection. All recipients were followed and the postoperative progression was monitored by imaging. According to the patient's postoperative progression-free survival time, X-tile software was used to calculate the clinical prognosis value of the PAC-1$^+$, CD62p$^+$ and PAC-1$^+$CD62p$^+$ percentages. The cut-off values of PAC-1$^+$, CD62p$^+$ and PAC-1$^+$CD62p$^+$ percentages for prognosis were 17.2%, 14.6% and 10.2%, respectively.

3.3. Prognostic value of platelet markers in patients treated with TACE

In the TACE group, patients were divided into 2 groups based on the cut-point values obtained from X-tile software. In the groups with a higher marker-positive percentage of platelets, the percentage of patients with final postoperative tumor progression increased (PAC-1$^+$, 80.00% for > 17.2% vs 62.22% for ≤17.2%, p = .018; CD62p$^+$, 82.25% for > 14.6% vs 65.63% for ≤14.6%, p = .015; PAC-1$^+$CD62p$^+$, 81.49% for > 10.2% vs 68.00% for ≤10.2%, p = .032) (Fig. 3A). Similarly, in the Kaplan-Meier analysis, the patients with a higher marker-positive percentage of platelets had shorter progression-free time (p = .003 for PAC-1$^+$, p = .038 for CD62p$^+$, p = .044 for PAC-1$^+$CD62p$^+$) (Fig. 3B).

In univariate COX regression analysis, the PAC-1$^+$ percentage (hazard ratio [HR], 1.867; 95% CI, 1.208–2.865; p = .005) and CD62p$^+$ percentage (HR, 1.516; 95% CI, 1.010–2.275; p = .044) were the risk factors for prognosis as well as the number of tumors (HR, 1.992; 95% CI, 1.107–3.586; p = .022) and AFP (HR, 1.268; 95% CI, 1.112–1.756; p = .041) (Table 3). The multivariate regression analysis showed that the PAC-1$^+$ percentage (HR, 1.834; 95% CI, 1.098–3.091; p = .020) was an independent risk factor for the postoperative prognosis of HCC patients treated with TACE.

3.4. Prognostic value of platelet markers in patients undergoing resection

In the resection group, patients were also divided into 2 groups based on the cut-point values. In the groups with a low marker-positive percentage of platelets, the percentages of patients with final postoperative tumor progression were 54.00% (for PAC-1$^+$), 54.16% (for CD62p$^+$) and 64.70% (for PAC-1$^+$CD62p$^+$), respectively (Fig. 4A). Meanwhile, in the groups with a higher marker-positive percentage of platelets, the percentages of patients with final postoperative tumor progression were 91.90% (for PAC-1$^+$), 84.61% (for CD62p$^+$) and 82.14% (for PAC-1$^+$CD62p$^+$), showing a significant increase (p < .001 for PAC-1$^+$, p = .001 for CD62p$^+$, p = .010 for PAC-1$^+$CD62p$^+$). In addition, the Kaplan-Meier analysis of progression-free time showed that the high marker-positive percentage indicated poor prognosis (p = .001 for PAC-1$^+$, p = .005 for CD62p$^+$, p = .044 for PAC-1$^+$CD62p$^+$) (Fig. 4B).

For patients treated with resection, the univariate COX regression analysis showed that the risk factors for poor prognosis included the PAC-1$^+$ percentage (HR, 6.166; 95% CI, 2.961–12.841; p = .000), the CD62p$^+$ percentage (HR, 2.322; 95% CI, 1.211–4.451; p = .011), the number of tumors (HR, 3.558; 95% CI, 1.915–6.610; p = .000), portal vessel invasion (HR, 3.557; 95% CI, 1.736–7.289; p = .001) and Barcelona clinic liver cancer staging (BCLC, HR, 3.053; 95% CI, 1.356–6.876; p = .007) (Table 4). Although some risk factors were different from those for TACE, the importance of the PAC-1$^+$ and CD62p$^+$ percentages of platelets was consistent. The multivariate regression analysis showed that the PAC-1$^+$ percentage (HR, 4.756; 95% CI, 1.813–12.477; p = .002) was also an independent risk factor for the postoperative prognosis of HCC patients treated with resection.

4. Discussion

We established a platelet function test on flow cytometry to determine the platelet activation using 2 markers, PAC-1 and CD62p. By this test, we investigated clinical implications of platelet function in HCC. We enrolled 2 groups of HCC patients, surgical resection and TACE groups, as well as healthy people. We defined 3 different groups of platelets according to platelet markers. The 3 groups are PAC-1$^+$, CD62p$^+$ and PAC-1$^+$CD62p$^+$. The clinical value of these 3 platelet markers in the diagnosis and postoperative prognosis of HCC was evaluated.

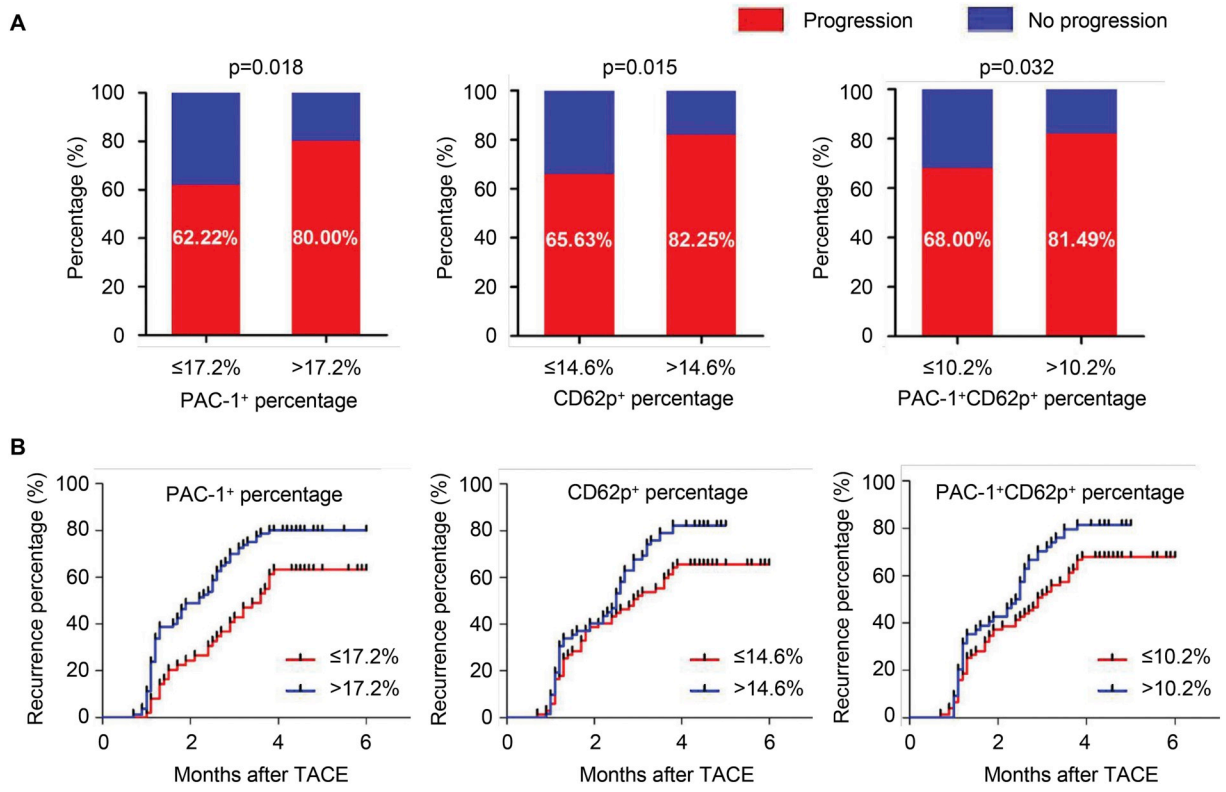

Fig. 3. Platelet markers in the prognosis of the HCC patients treated with TACE. (A) Final tumor progression in the HCC patients with different levels of platelet markers. The *p* values are shown. (B) Kaplan-Meier analysis of tumor recurrence in the HCC patients with different levels of platelet markers.

Table 3
Univariate and multivariate COX regression analysis of prognosis after TACE.

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Age, > 50 vs ≤50 y	0.832 (0.533–1.301)	NS	NA	NA
Sex, male vs female	1.725 (0.895–3.326)	NS	NA	NA
HBs Ag, positive or negative	0.936 (0.471–1.862)	NS	NA	NA
Liver cirrhosis, Child B-C vs Child 0-A	0.367 (0.051–2.638)	NS	NA	NA
Tumor Number,Mulitple vs Single	**1.992 (1.107–3.586)**	**0.022**	**2.369 (1.234–4.545)**	**0.009**
AFP, > 400 vs ≤**400 ng/ml**	**1.268 (1.112–1.756)**	**0.041**	1.259 (0.827–1.928)	NS
Tumor size, > 5 cm vs ≤ 5 cm	1.763 (0.853–3.642)	NS	NA	NA
Portal vessel invasion, yes vs no	1.218 (0.813–1.826)	NS	NA	NA
BCLC,B-D vs 0-A	0.636 (0.277–1.458)	NS	NA	NA
PLT, > 222 × 10^9/L vs ≤222 × 10^9/L	**2.499 (1.254–4.982)**	**0.009**	**2.082 (1.022–4.245)**	**0.043**
PAC-1^+, > 17.2% vs ≤17.2%	**1.867 (1.208–2.865)**	**0.005**	**1.834 (1.098–3.091)**	**0.020**
$CD62p^+$, > 14.6% vs ≤14.6%	**1.516 (1.010–2.275)**	**0.044**	1.266 (0.797–2.015)	NS
PAC-1^+CD62p^+, > 10.2% vs ≤10.2%	1.498 (0.999–2.248)	NS	NA	NA

NA, not applicable. NS, not significant.

The results showed that the levels of PAC-1^+, $CD62p^+$ and PAC-1^+CD62p^+ platelets in peripheral blood of patients with HCC were significantly higher than those of healthy people, indicating that there was significant abnormal activation of peripheral platelets in patients with HCC. The percentages of PAC-1^+, $CD62p^+$, PAC-1^+CD62p^+ groups were analyzed statistically in healthy people and HCC patients to evaluate their value in the diagnosis of HCC (Fig. 2 and Table 1). Among them, PAC-1^+ percentage has the best diagnostic efficiency, while $CD62p^+$ and PAC-1^+CD62p^+ percentages are slightly weaker. As a platelet membrane marker, the sensitivity of PAC-1 was inferior to AFP in the diagnosis of HCC, so we combined PAC-1 with AFP as a new marker and found it diagnostic efficiency was significantly improved.

In the prognosis evaluation of patients with HCC, we used X-tile software combined with the disease-free survival of patients to predict the cut-point values of these 3 markers. In the TACE group, the Kaplan-Meier analysis showed that all 3 marker-positive percentages were meaningful in the prognosis of patients ($p < .05$). In COX regression analysis, the number of tumors, the number of platelets, and PAC-1^+ percentage were independent risk factors for the prognosis of patients undergoing TACE. In the surgical resection group, similar results were produced. The Kaplan-Meier analysis revealed that the 3 marker-positive percentages were also meaningful in the prognosis of patients ($p < .05$) and the COX regression analysis showed that the number of tumors and PAC-1^+ percentage were independent risk factors for the prognosis of patients undergoing resection. In summary, we can conclude that PAC-1^+ percentage of platelets has significance in the diagnosis and prognosis of HCC patients. It could be a marker for effective prognosis of HCC in potential. At the same time, we also concluded that the abnormal early activation of platelets is a risk factor for HCC diagnosis and prognosis.

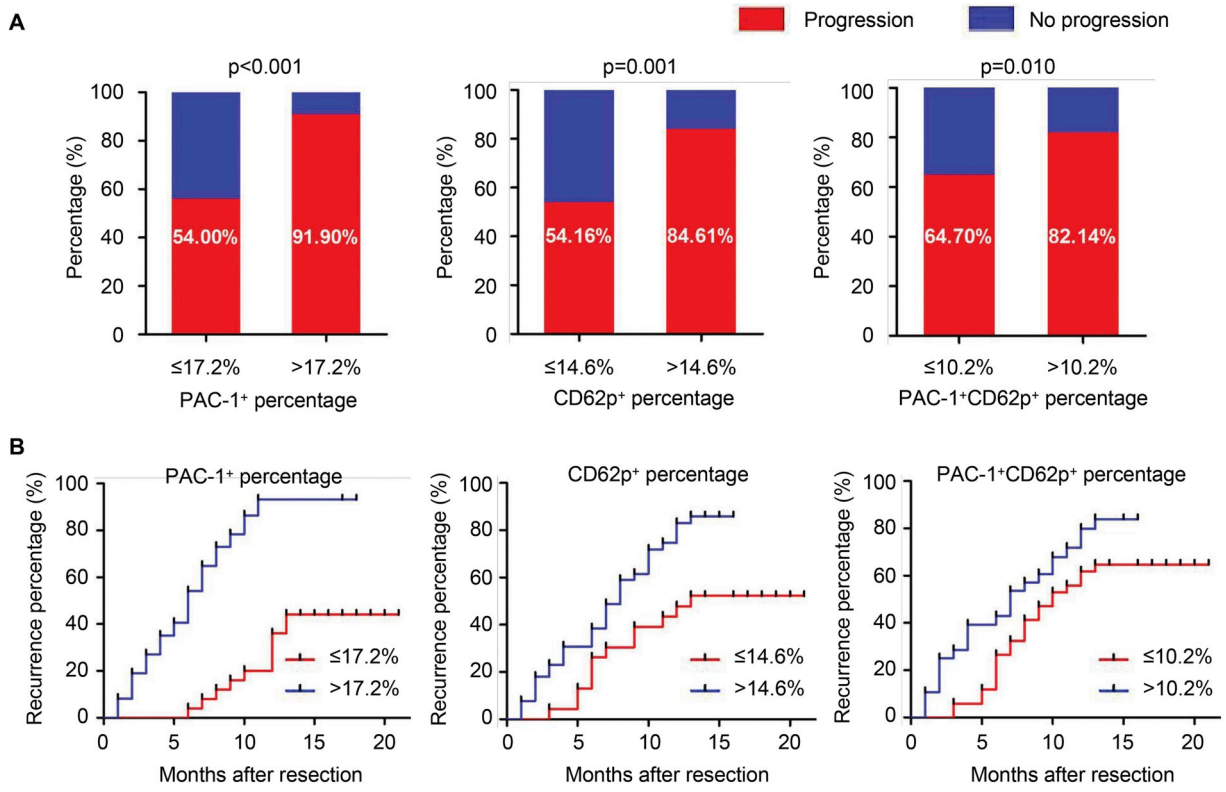

Fig. 4. Platelet markers in the prognosis of the HCC patients undergoing resection. (A) Final tumor progression in the HCC patients. The patients were divided into 2 groups based on the levels of platelet markers. The p values are labeled. (B) Kaplan-Meier analysis of tumor recurrence in the 2 groups of HCC patients.

Table 4
Univariate and multivariate COX regression analysis of prognosis after resection.

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Age, > 50 vs ≤50 Y	0.842 (0.465–1.522)	NS	NA	NA
Sex, male vs female	2.115 (0.754–5.927)	NS	NA	NA
HBs Ag, positive or negative	0.570 (0.298–1.087)	NS	NA	NA
Liver cirrhosis, Child B-C vs Child 0-A	0.676 (0.358–1.274)	NS	NA	NA
Tumor Number,Mulitple vs Single	**3.558 (1.915–6.610)**	**0.000**	**2.692 (1.332–5.441)**	**0.006**
AFP, > 400 vs ≤**400 ng/ml**	0.709 (0.381–1.320)	NS	NA	NA
Tumor size, > 5 cm vs ≤5 cm	1.566 (0.834–2.904)	NS	NA	NA
Portal vessel invasion, yes vs no	**3.557 (1.736–7.289)**	**0.001**	1.726 (0.741–4.021)	NS
BCLC,B-D vs 0-A	**3.053 (1.356–6.876)**	**0.007**	1.308 (0.512–3.341)	NS
PLT, > 222×10^9/L vs ≤ 222×10^9/L	**1.887 (0.837–4.257)**	**NS**	NA	NA
PAC-1^+, > 17.2% vs PAC-1^+ ≤ 17.2%	**6.166 (2.961–12.841)**	**0.000**	**4.756 (1.813–12.477)**	**0.002**
$CD62p^+$, > 14.6% vs $CD62p^+$ ≤ 14.6%	**2.322 (1.211–4.451)**	**0.011**	1.543 (0.789–3.018)	NS
PAC-1^+CD62p^+, > 10.2% vs ≤10.2%	1.771 (0.985–3.183)	NS	NA	NA

NA, not applicable, NS, not significant.

Experimental reports and animal models had demonstrated that platelets, as an important component of the tumor tissue microenvironment [11,12], play an important role in helping tumor tissues escape immune surveillance and post-treatment metastasis and recurrence [13]. Activated platelets protect tumor cells by adhering detached tumor cells to the vessel wall or encapsulating them, inducing recurrence and distant metastasis in tumor patients [14]. In addition, there are also reports that activated platelets secrete a variety of substances, which can cause various functional changes of tumor cells, and also cause changes in the body itself, including angiogenesis, inflammation and so on [15]. These findings may explain why platelet activation status has diagnostic and prognostic values.

Through the detection of peripheral platelet markers and population comparisons, we found that the early activation marker of platelet, PAC-1, can effectively assist clinical diagnosis of HCC and determine the prognosis of patients after operation. However, there are also shortcomings. First, the follow-up time of the population is short. Secondly, the number of patients enrolled in the resection group was small. The population should be continuously supplemented in the later study to obtain more detailed statistical analysis.

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