

## Original Article

# Platelets and podoplanin is prognostic of patient survival after surgery in colorectal cancers

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**Abstract:** Podoplanin expressing cancer-associated fibroblasts (CAFs) and high preoperative platelet count are known to be associated with tumor microenvironment that ultimately affect tumorigenesis. However, whether these two factors could serve as prognostic biomarkers in colorectal cancer remains unknown. This study explores the role of podoplanin-expressing cancer-associated fibroblasts, platelet count (PLT) and platelet-to-lymphocyte ratio (PLR) in predicting colorectal cancer survival. PDPN-expressing cancer-associated fibroblasts and platelets infiltration in tumor tissues of 164 cases of colorectal cancer patients were measured by immunohistochemistry staining. The data of preoperative platelet count and PLR were collected. Patients with podoplanin-expressing cancer-associated fibroblasts or preoperative PLR less than the median had better overall survival compared with the control group ( $P=0.004$  or  $0.003$ ). Multivariate analysis showed that preoperative PLR  $\geq$  median was an independent risk factor for the prognosis of colorectal cancer (HR: 2.775; 95% CI: 1.50-5.14;  $P=0.01$ ). Platelets infiltration into tumor tissue were closely associated with podoplanin expression in CAFs, but showed no correlation with peripheral blood platelets. The results show that CAFs with podoplanin-expression were found to be a good prognosis factor for colorectal cancer patients. However, evidence supporting the association between platelet infiltration and prognosis of colorectal cancer is lacking, which requires further studies in the future.

**Keywords:** Colorectal cancer, cancer associated fibroblasts, podoplanin, infiltration of platelets, prognosis

## Introduction

Colorectal cancer (CRC) is one of the major causes of morbidity and mortality worldwide. Though a relatively lower incidence and death rate were observed in Asia populations [1], the incidence of CRC in China has a rapid growth due to the increase in aging population, deterioration of the environment, and adoption of western lifestyle [2]. Meanwhile, the 5-year survival rate of CRC in China has not been improved a lot [3]. Therefore, there is an urgent need to identify molecular markers to avail prognosis before or after the surgery by complementing histopathological staging.

Tumor micro-environment (TME) is the cellular environment composed of non-cancer cells and their stroma such as blood vessels, immune cells, fibroblasts, and extracellular matrix [4]. A number of stromal cells are recruited to TME to establish a suitable environment to pro-

mote the growth and metastatic dissemination of tumor cells [5, 6]. Among the stromal cells, activated fibroblasts which are found in association with cancer cells are called cancer-associated fibroblasts (CAFs). CAFs play an important role in the development and progression of tumors by secreting growth factors and cytokines surrounding the cancer cells, which are involved in the formation of blood vessels [7].

Podoplanin (PDPN) is a specific marker for lymphatic endothelium which is widely used in histopathology. However, recent studies reported that PDPN expression in CAFs is associated with tumor progression and predicts a worse prognosis in patients carrying different cancers [8-10].

In this study, we examined PDPN expression in CAFs and the infiltration of platelet in tumor tissues by immunohistochemistry staining in 164 tumor samples of CRC patients. Combined wi-

**Table 1.** Clinicopathological characteristics of patients carrying colorectal cancer

Group	EGTM of PLT (n=82)	EGTM of PLR (n=82)	PDPN+ (n=98)	CD61+ (n=121)
Gender (Male/Female)	37/45	40/42	54/44	64/57
Average age	60.3	63.1	62.0	61.9
Rectum	36	34	64	54
Colon	46	48	66	67
Tumor long diameter (cm)	4.94	4.67	4.20	4.30
N Stage				
0	38	45	62	71
1	26	22	24	33
2	18	15	12	17
Clinical stage				
I	11	12	19	24
II	27	22	42	46
III	44	47	37	49
IV	0	1	0	2

The clinicopathological characteristics include tumor location, tumor size, lymph node metastasis and distant metastasis. There are 29, 62, 71, and 2 cases of TNM stages I to IV, respectively. Patients were tested by tumor markers (for example: CEA, CA199 and CA125) and computerized tomography (CT) scanned every three months within the first 2 years, twice per year during 2 to 5 years, and once per year after 5 years after surgery; and the colonoscopy was reviewed once per year. The follow-up data was collected from the date of surgery and updated in December 2014.

th the analysis of pre-operative platelet count (PLT) and platelet-to-lymphocyte ratio (PLR), we evaluated the predictive value of these parameters for the prognosis of patients with colorectal cancer, and analyzed the correlation between the peripheral blood PLT and the infiltration of platelet in tumor tissues.

**Patients and methods**

*Patients and tissue samples*

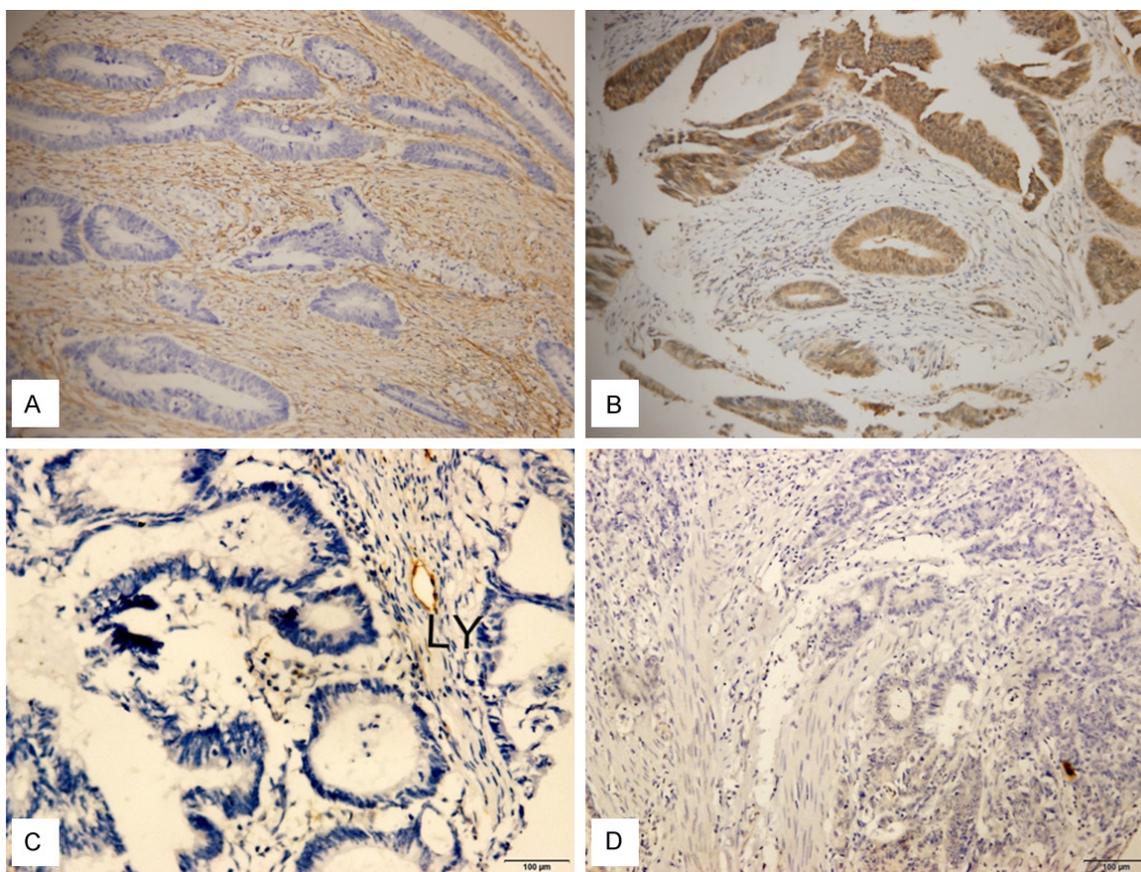
Patients were collected between January 2007 and December 2008 from the Affiliated Hospital of Jiangnan University at the time of primary surgery for suspected or confirmed cases of CRC. This study was approved by the Regional Ethics Committee and the informed consent was obtained from the patients. Patients with pre-operative mortality, co-occurrence with other autoimmune diseases, and incomplete information were excluded from the statistical analysis. The total study population was 164 patients, with 76 of the tumor localized in the rectum and 88 in the colon. Among them, 86 were males, 78 were females, and the average age was 62.0 ± 10.6 years. The complete blood count (CBC) data was collected at 1 week before surgery to compute PLR. The median of pre-operative PLT and PLR were 206×10<sup>9</sup>/L and 136×10<sup>9</sup>/L, respectively. The patients were stratified into two groups by the median, i.e, less than median group (LTM) and equal or greater than the median group (EGTM).

*Immunohistochemistry*

Sections of formalin-fixed, paraffin-embedded tissue were immunostained using the Dako En-Vision method (Dako, Glostrup, Denmark) and mouse monoclonal anti-human CD61 (Klon SZ-21, gift from Thrombosis and Haemostasis Research Unit, Jiangsu Institute of Hematology) and anti-PDPN antibodies (clone: D2-40, DAKO). A specimen was considered positive for PDPN expression in CAFs (PDPN+) when distinct staining was observed in ≥ 10% of the fibromatous tumor stroma. The negative control was obtained by replacing the primary antibody with normal monoclonal mouse IgG of the same subclass and concentration. Scattered punctate staining around tumor cells was considered positive for infiltration of PLT into tumors (CD61+), and PLT in the arterial infiltration was used as the internal positive control.

*Statistical analysis*

To evaluate the associations between PDPN or CD61 expression and clinicopathological parameters, we stratified PDPN or CD61 tumor grades into negative and positive stainings. The associations were tested using two-tailed Fisher’s exact test or Chi-squared test. Univariate survival analysis was performed using the log rank test. Multivariate survival analysis was conducted using the Cox proportional hazards regression model, and the variables were optimized using the backward, stepwise elimination



**Figure 1.** Immunochemical staining of CRCs showing PDPN expression in CAFs and tumor infiltration of platelets. A. Colorectal cancer samples with high amount of PDPN-expressing CAFs. B. Samples with infiltration of platelets around the tumor cells. C. Samples without PDPN-expressing CAFs. 'LY' denotes PDPN+ peritumoral lymphatic vessels. D. Samples without infiltration of platelets around the tumor cells.

approach. Data analysis was performed using SPSS version 17.0 (SPSS, Chigaco, IL). Statistics with  $P < 0.05$  were considered statistically significant.

## Results

### *PDPN expression in patient samples*

The clinical and pathological features of the patient cohort under study are summarized in (**Table 1**). The average age of patients at the time of surgery was 62 years old. The majority of the patients showed PDPN+ ( $n=98$ ) or CD61+ ( $n=121$ ). The clinical and pathological features showed no significant difference among the four studied groups, i.e, EGTM of PLT group, EGTM of PLR group, PDPN+ group, and CD61+ group.

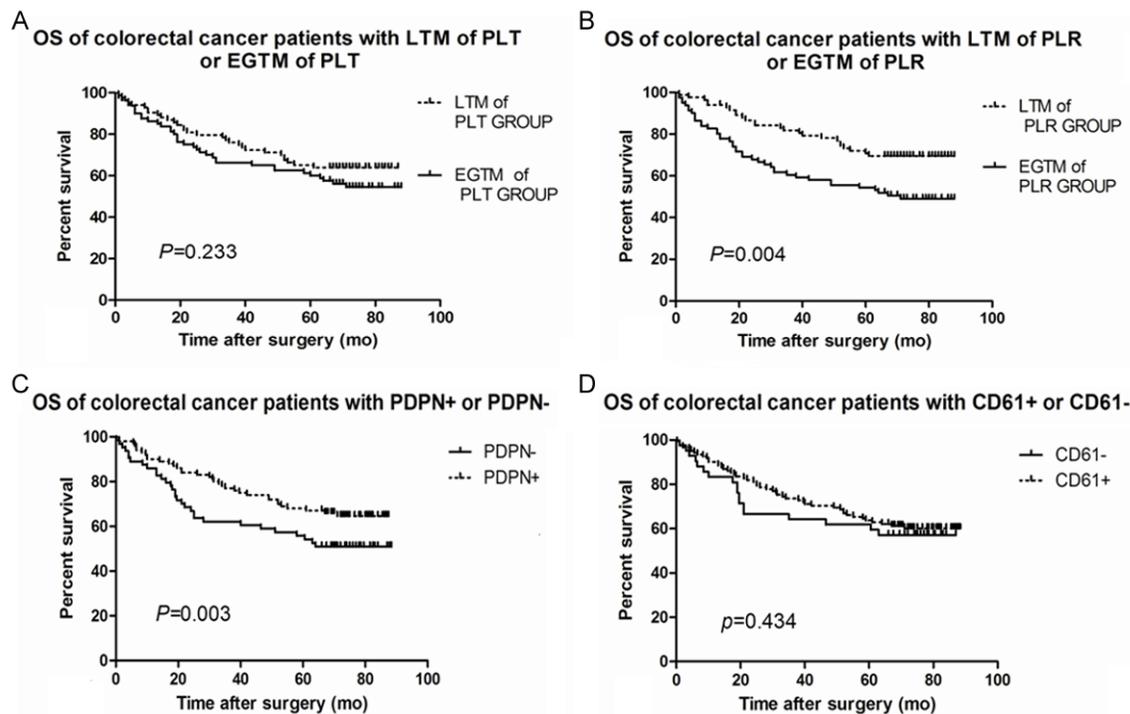
### *PDPN expression in CAFs and tumor infiltration of platelets*

PDPN-expressing CAFs were exclusively present in tumor stroma and easily identified as

large spindle-shaped mesenchymal cells with stress fibers and well-developed fibronexus (**Figure 1A**). 98 samples (60%) were considered PDPN+ because at least 10% of CAFs showed a distinct staining pattern. While in samples without PDPN-expressing CAFs, PDPN-positive peritumoral lymphatic vessels were observed (**Figure 1C**). Infiltrating platelets were exclusively detected in the surrounding region of tumor cells but not within tumor cells or tumor stroma. 121 samples (73.8%) were considered CD61+ with distinct staining pattern. The infiltrated area of the platelets was not as wide as that of PDPN-expressing CAFs (**Figure 1B, 1D**).

### *Correlation between clinical pathological parameters and the four groups*

According to two-tailed Fisher's exact test, significant association was observed between PDPN+ and clinical factors, including clinical stage ( $P=0.048$ ), N stage ( $P=0.018$ ), and T



**Figure 2.** Kaplan Meier curves on CRC patient OS as stratified by follow figures. A. LTM of PLT group and EGTM of PLT group. B. LTM of PLR group and EGTM of PLR group. C. PDPN+ or PDPN- expressing CAFs. D. CD61+ or CD61- infiltration of platelets into tumors.

stage ( $P=0.021$ ) in CRC patients. A significant association was also observed between PDPN+ and CD61+ ( $P=0.001$ ). However, no obvious correlation was observed between the pre-operative PLT or PLR value with PDPN or CD61 expression in tumor tissues.

*Prognostic significance of PDPN-expressing CAFs and tumor infiltration of platelets*

Using log rank test, we found that PLR and PDPN are prognostic of patient OS. The LTM of the PLR group had significantly increased OS as compared with EGTM of the PLR group ( $P=0.004$ ) (Figure 2B), and no significant association was observed between PLT and OS (Figure 2A). A significantly more favorable OS was observed for PDPN+ patients as compared with PDPN- patients ( $P=0.003$ ) (Figure 2C), whereas no significance was observed for patients stratified by CD61 status (Figure 2D). We performed the multivariate analysis using the Cox regression model, where variables T stage, N stage, TNM stage, EGTM of PLT, EGTM of PLR, and PDPN+ and CD61+ were included. The results displayed that the TNM stage (I II/III IV) (HR: 0.657; 95% CI: 1.31-19.22;  $P=0.041$ ), T stage (T1-2/

T3-4) (HR: 2.857; 95% CI: 1.32-6.15;  $P=0.007$ ) and EGTM of PLR (HR: 2.775; 95% CI: 1.50-5.14;  $P=0.011$ ) are independent risk factors of OS for CRC patients (Table 2).

**Discussion**

CRC is the most common type of gastrointestinal cancers, whose occurrence is influenced by genetic factors, environmental exposures, and inflammatory conditions of the digestive tract [11]. With alterations in the life style, CRC incidence rapidly increased during the past few years especially in China. Identifying novel prognostic markers of cancer progression is urgently needed despite the extensive basic and clinical research on CRC. Though genetic alterations in tumor cells is an important factor driving tumorigenesis [12], tumor stromal cells play an important role in this process [13-15]. CAFs constitute one type of important stromal cells, which secrete angiogenic factors, matrix metalloproteinases, and inflammatory chemokines to promote tumor growth, invasion and metastasis [16-19].

The prognostic role of PDPN on cancers varies with cancer types. For instance, PDPN-express-

**Table 2.** Univariate and multivariate analyses on CRC patient OS

Risk factors	Univariate		Multivariable	
	P value	P value	HR	95% CI
PDPN+	0.003*	0.123	0.646	0.37-1.12
CD61+	0.398	0.717	1.114	0.62-2.0
T stage (T1-2/T3-4)	0.002*	0.007*	2.857	1.32-6.15
N stage (N0/N1-2)	0.001*	0.825	1.265	0.16-10.1
TNM stage (I II/III IV)	0.001*	0.041*	2.428	1.31-19.22
EGTM of PLR	0.004*	0.011*	2.775	1.50-5.14
EGTM of PLT	0.233	0.091	0.582	0.31-1.08

HR; Hazard ratio, \*P<0.05.

ing CAFs were significantly associated with the progression of lung adenocarcinoma [8, 20], ovarian cancer [21] and invasive ductal carcinoma [9]. In contrast, it is an independent predictor of good outcome of colorectal cancer [10], lung squamous cell carcinoma [22] and cervical cancer [23].

The dissemination of malignant cells into blood and their transportation play a pivotal role in cancer development which involves a wide spectrum of blood-related cells such as PLT, lymphocytes, PLR and macrophages [15]. Of the many blood cells associated with cancer metastasis, PLT and PLR are relatively well studied [24, 25]. A high level of PLT has been associated with advanced stage and death for several malignancies including CRC [26, 27], and PLT infiltration into tumors has been reported to promote tumor progression [28].

PDPN can induce PLT aggregation by interacting with its counterpart CLEC-2, which is expressed on PLT surfaces. Activated PLT can secrete growth factors to enhance the growth and motility of primary tumors and tumor vasculature [29]. However, no study has reported the association between PDPN-expressing CAFs and PLT infiltration in CRCs. Thus, we investigated the correlation of these two factors with clinical pathological parameters in patients carrying CRC. By immunohistochemistry analysis, we confirmed that PDPN+ is significantly associated with TNM stage, N stage and T stage, supporting its involvement in CRC development. We also found a significant association between PDPN+ and CD61+. However, CD61+ has no association with pre-operative PLT, PLR or TNM stage.

The prognostic significance of PDPN+ and CD61+ was subsequently assessed by log rank test and Cox proportional hazards regression

model. The results revealed that PDPN+ was associated with favorable patient OS, which is in accordance with a recent report by Yamanashi T et al [10]. CD61+ showed a trend towards increased survival. Both clinical factors were not independent prognostic factors for the OS of CRC patients.

In conclusion, we found that platelets can be infiltrated into tumor tissue of CRC, but there were no correlation between the pre-operative PLT or PLR value with PDPN or CD61 expression in CRC patients. We further confirmed that EGTM of PLR has significantly shorter OS time than that of LTM of PLR, and PLR is an independent prognostic factor. We demonstrated that PDPN+ predicts good outcome in CRCs. PDPN+ showed significant association with CD61+, but these PLT did not show any influence in the prognosis of CRCs, which might be caused by the barrier of adventitia with adenoid structure. PDPN+ and CD61+ were not correlated with prognosis in multivariate regression analysis, which might be due to the limited cases and follow-up time and needs to be verified in an expanded study. Our findings collectively demonstrate that PDPN-expressing CAFs and pre-operative PLR can be potentially used in clinics to avail accurate CRC patient stratification and treatment decision making.

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**Disclosure of conflict of interest**

None.

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## Microenvironment of tumor in CRC

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