RESEARCH ARTICLE

Elevated Platelets Enhance Cancer Cell Migration, Promote Hematogenous Metastasis and Associate with a Poor Prognosis in Advanced Non-small Cell Lung Cancer Cases

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Abstract

Although correlations between platelets and lung cancer has been recognized, effects on non-small cell lung cancer (NSCLC) metastasis remain to be determined in detail. In the present study, wound healing assays revealed a role of platelets in NSCLC cell migration . Thus the mean migration rate of lung adenocarcinoma A549 cells was significantly elevated after co-culture with platelets (81.7±0.45% vs 41.0±3.50%, *P*<0.01). Expression of GAPDH was examined by reverse transcription-polymerase chain reaction to study the effect of platelets on NSCLC cell proliferation. The result showed that the proliferation of A549 and SPC-A1 cells was not affected. Mouse models were established by transfusing A549 cells and SPC-A1 cells into mice lateral tail veins. We found tumor metastasis nodules in lungs to be increased significantly after co-transfusion with platelets (in A549, 4.33±0.33 vs 0.33±0.33, *P*=0.01; in SPC-A1, 2.67±0.33 vs 0.00±0.00, *P*=0.01). In addition, consecutive inoperable patients with newly diagnosed NSCLC (TNM stage III or IV) between January 2009 and December 2011 were retrospectively reviewed. Using the Kaplan-Meier method, NSCLC patients with a high platelet counts demonstrated a significantly shorter progression free survival compared with those with a low platelet count (>200×10^9/L, 3 months versus ≤200×10^9/L, 5 months, *P*=0.001). An elevated platelet count was also identified as an independent prognostic factor by Cox regression analysis for prgression free survival (adjusted hazard ratio: 1.69; 95% CI: 1.16, 2.46; *P*=0.006). This study suggested that platelets might contribute to the hematogenous metastatic process by promoting cancer cell migration, which eventually affects the prognosis of NSCLC.

Keywords: Platelets - non-small cell lung carcinoma - metastasis - survival - prognosis

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Introduction

Lung cancer has been one of the leading causes of cancer mortality worldwide (Siegel et al., 2012). Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases, with a five-year survival rate of 15.1% in China (Herbst et al., 2008). The main death cause of NSCLC is the cancer metastasis which has being the difficulty of treatment and hotspot of lung cancer research. It is urgent for clinicians and researchers to explore treatment targets to inhibit cancer metastasis thereby establishing more reliable and effective therapies for NSCLC.

Platelets are small, specialized blood cells that are released as anuclear cytoplasmic bodies from megakaryocytes in the bone marrow. The main function of platelets is to halt hemorrhage after tissue trauma and vascular injury (Ruggeri et al., 2007; Davì et al., 2007). The contribution of platelets to the cancer metastasis has been an emerging area of research interest. High platelet count has been demonstrated associated with poor prognosis in many different tumor entities, including endometrial carcinoma (Scholz et al., 2000; Ayhan et al., 2006), cervical cancer (Hernandez et al., 1992), ovarian cancer (Zeimet et al., 1994), gastric cancer (Ikeda et al., 2002), and esophageal cancer (Shimada et al., 2004). Regarding NSCLC, Costantini et al demonstrated that NSCLC patients had increased prevalence of thrombocytosis and relative high platelet levels during disease progression (Costantini et al., 1990). Among operable I-III NSCLC patients, those with elevated platelet counts showed an increased risk of disease progression and overall survival (Tomita et al., 2008; Yu et al., 2013). Thrombocytosis was also associated with overall survival in III/IV NSCLC patients (Engan and Hannisdal, 1990). Thrombocytosis were more seen in lung cancer patients with advanced TNM stages (Pedersen and Milman, 1996). However, whether and how the platelets affect the metastatic process of NSCLC has not been fully studied.

The aim of the present study was to investigate the effects of platelets on NSCLC cell proliferation and migration. Furthermore, the role of platelets in NSCLC

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hematogenous metastasis was studied. The association of platelet count with progression free survival in TNM stage III/IV NSCLC patients was also examined.

Materials and Methods

Cell lines

Human lung adenocarcinoma cell lines A549 and SPC-A1 were purchased from Shanghai institute of biochemistry and cell biology (Shanghai, China). The two cell lines were maintained in laboratory and grown in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 100 U/ml Penicillin, 100 μg/ml Streptomycin and 10% fetal bovine serum (FBS). All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

Platelets isolation

Platelet-rich plasma (PRP) was obtained from supernatant of human whole blood by centrifugation at 150×g for 15 min. Washed platelets were prepared from pellets of PRP by centrifugation at 900×g for 5 min following washing with modified Tyrode's buffer (20mM HEPES, 150 mM NaCl, 2.5 mM KCl, 12 mM $NaHCO_3$, 1mg/ml of glucose, 1mM $MgCl_2$, and 1mg/ml BSA), supplemented with 2μmol/L prostaglandin-E. Before following experiments, washed platelets were resuspended in modified Tyrode buffer and incubated for 30 min at 37°C, which was designated as PLT group. For control, platelets were prefixed in formalin and washed three times with Tyrode's buffer and this group was defined as PLT/D compared with PLT group.

Wound healing assay

Cells were seeded into 12-well plates and cultured overnight to form confluent monolayer. After scratched with a sterile pipette tip, cells were rinsed gently with PBS to remove the detached cells and incubated with medium containing 1% FBS and platelets (1×10^7/ml) at 37°C in an atmosphere of 5% CO_2. The image of wounded areas was taken at 0h and 24h after incubation. The distances between the two edges of the scratched cells were measured and healing rate was calculated using the following formula: healing rate= (the distance prior to healing- the distance following healing)/the distance prior to healing×100%.

Reverse transcription-polymerase chain reaction (RT-PCR)

To further study the effect of platelets on lung cancer cell proliferation, the expression of GAPDH by RT-PCR was examined. Equal number cells (1×10^5/ml) were seeded into 6-well plates, and cultured with medium containing 10% FBS and platelets (1×10^7/ml). Total RNA was extracted with TRIzol reagent (Invitrogen, USA) and the concentration of total RNA was measured by spectrophotometer (BioPhotometer, Eppendorf, Germany). PrimeScriptTM 1st Strand cDNA Synthesis Kit (TaKaRa Bio Inc., Japan) and GoTaq® Colorless Master Mix (Promega, USA) were used for first strand cDNA synthesis and thereafter PCR. All the operations were in accordance with the manufacturer's instructions. A total of 2 ug RNA was tipped out for cDNA synthesis and 25 ul reaction volume was fixed for all further PCR. We evaluated the expression of GAPDH with following primers as forward 5'-CAATGACCCCTTCATTGACC-3'; reverse 5'-TGGAAGATGGTGATGGGATT-3'. The PCR products were electrophoresed on 2% agarose gels and detected by ethidium bromide staining. Images were obtained and the gray values of all the products were measured by ImageJ (http://rsbweb.nih.gov/ij/).

Animal models

BALB/C nude mice (6-8 weeks old) were purchased from the Academy of Military Medical Sciences (Beijing, China). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and bred under controlled temperature and humidity, and a 12-hours dark, 12-hours light cycle with sterile food and water ad libitum. Lung adenocarcinoma hematogenous metastasis models were established by transfusing A549 cells and SPC-A1 cells (1×10^6/100μl PBS) from mice lateral tail vein. To study the effect of platelets on lung adenocarcinoma hematogenous metastasis, 1×10^7/ml platelets were added to A549 cell and SPC-A1 cell suspension before transfusion, with formalin fixed platelets as control. Five mice were included in each group and the experiment was repeated twice. Whole lung tissue was obtained 4 weeks later and fixed in Bouin's Fixative and embedded in paraffin blocks. Continuous section slides were made and stained by H&E. Tumor metastasis nodules were counted under microscope. Images were acquired on Olympus BX-60 light microscope. Animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Ethics Review Board for Animal Studies of Nanjing Drum Tower Hospital (DTH ERBA 66.01/023B/2011).

Patients

Consecutive patients with newly diagnosed NSCLC between January 2009 and December 2011 from the Affiliated Drum Tower Hospital of Nanjing University Medical School (Nanjing, China) were retrospectively reviewed. All included patients were TNM stage III or IV who had been assessed inoperable thus did not receive lung resection afterwards. They all received chemotherapy for NSCLC in our hospital. Full medical record abstraction was conducted to obtain the following patient variables: platelet count, age, gender, cell type, tumor differentiation, and TNM stage. Specifically, only platelet count determined before invasive diagnostic procedures obtained at the first visit to our hospital was considered. Platelet count was determined by automated complete blood cell counters from ethylenediamine tetra-acetic acid (EDTA)-anticoagulated blood samples in our clinical laboratory. A total of 126 patients with complete data were identified in the current study. Their contact materials and the study protocol were reviewed and approved by the Drum Tower Hospital Institutional Review Board (Nanjing, China).

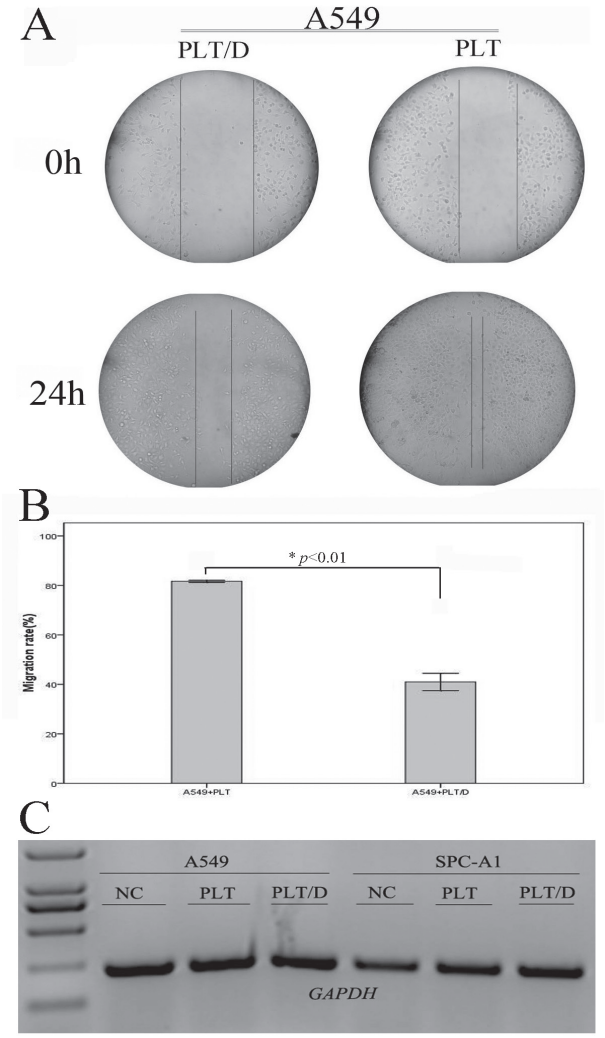

Figure 1. Platelets Promoted the Migration of Lung Cancer Cells. A, Wound healing assay, A549 cells were seeded into 12-well plates and cultured overnight to form confluent monolayer. After scratched with a sterile pipette tip, cells were rinsed gently with PBS to remove the detached cells and incubated with medium containing platelets (1×10^7/ml) with formalin fixed platelets as control. The photos were captured at 0h and 24h after co-culture and at the same point. B, Migration rate of A549 cells in different groups. Scale bar, SEM. *P<0.01. C, Platelets co-culture did not affect the proliferation of A549 and SPC-A1 cells, as represented by expression of GAPDH. PLT, platelets; PLT/D, formalin fixed platelets as control

Statistical methods

Data from in vitro studies were expressed as mean ± S.D. and statistical significance was assessed by the Student's t-test. The progression (local progression and distant metastasis) of NSCLC was confirmed by radiology examination. Progression free survival (PFS) was calculated as the period from NSCLC diagnosis to disease progression or death by any cause. In our clinical laboratory, the normal range for platelet count is 100-300×10^9/L, thus the median 200×10^9/L was chosen as a cut off. The value of the platelet count was analyzed as a dichotomous variable (>200×10^9/L as high platelet group and ≤200×10^9/L as low platelet group). Associations between clinicopathological variables and platelet count were examined using a Pearson's χ^2 test for categorical variables (or Fisher's exact test if any sample number was less than 5), and a Student's t test for continuous variables. Univariate and multivariate Cox proportional hazards models were used to evaluate the prognostic impact of platelets on PFS. Kaplan-Meier analysis was performed for survival curve and statistical significance was assessed using the log-rank test. All analyses were performed with SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA). All tests were two-sided and performed at a significance level of 0.05.

Table 1. Characteristics of 126 Non-small Cell Lung Cancer Patients

Patient	Platelet count		*P* value
Characteristics	High >200×10^9/L (75) No. (%)	Low ≤200×10^9/L (51) No. (%)	
Age, years			0.79
Mean (SD[a])	57.4 (11.0)	56.9 (9.5)	
Gender			0.5
Female	31 (41.3)	18 (35.3)	
Male	44 (58.7)	33 (64.7)	
Cell type			0.3
Adenocarcinoma	61 (81.3)	45 (88.2)	
Squamous	14 (18.7)	6 (11.8)	
TNM stage			0.44
III	7 (9.3)	7 (13.7)	
IV	68 (90.7)	44 (86.3)	
Tumor differentiation			0.17
Well	2 (2.7)	3 (5.9)	
Moderately	12 (16.0)	14 (27.5)	
Poorly	61 (81.3)	34 (66.7)	

[a]standard deviation

Results

Platelets promoted the migration of lung cancer cells

After co-culture with platelets, lung adenocarcinoma cell A549 showed a significantly higher migration rate (81.7±0.45% vs 41.0±3.50%, P<0.01, Figure 1a and 1b). While the proliferation of the two lung adenocarcinoma cells, represented by expression of GAPDH, were not affected by platelets (Figure 1c).

Platelets increased hematogenous metastasis of lung cancer cells

The whole lung tissues of mice were sectioned and analyzed under microscopy. All the metastasis nodules were counted and summed. The results showed that platelets could increase the lung metastasis of the two lung adenocarcinoma cells, A549 and SPC-A1, as indicated in Figure 2. Tumor metastasis nodules increased significantly after co-transfusion with platelets (in A549, 4.33±0.33 vs 0.33±0.33, P=0.01; in SPC-A1, 2.67±0.33 vs 0.00±0.00, P=0.01).

Patients with high platelet count had poor prognosis

The median PFS of 126 NSCLC patients was 4 months (95% CI: 3.46, 4.54). A total of 75 patients (59.5%) had a platelet count more than 200×10^9/L. Clinicopathological characteristics of NSCLC are listed in Table 1 by platelet count. There were no significant differences in age, gender,

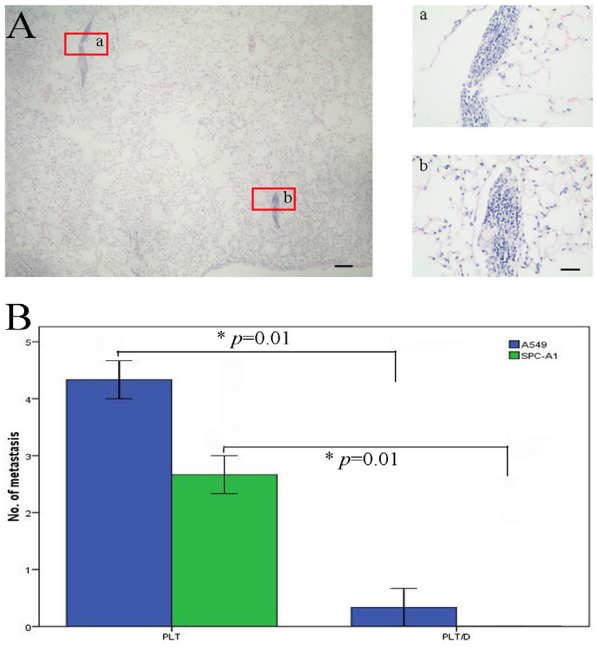

Figure 2. Platelets Increased Blood Metastasis of Lung Cancer Cells. A, Representative H&E photos of A549 cell lung metastasis nodules. Lung adenocarcinoma blood metastasis models were established by transfusion A549 cells and SPC-A1 cells (1×10^6/100μl PBS) from mice lateral tail vein. To study the effect of platelets on lung adenocarcinoma blood metastasis, 1×10^7/ml platelets were added to A549 and SPC-A1 cell suspension before transfusion, with formalin fixed platelets as control, n = 5. Scale bar, left 100μm, right 50μm; B, Total lung metastasis nodules of different groups. Whole lung tissue was obtained 4 weeks later and embedded in paraffin blocks. Continuous section slides were made and stained by H&E. Tumor metastasis nodules were counted under microscope. Co-transfusion of platelets could increase the lung metastasis of A549 and SPC-A1 cell. Scale bar, SEM. *P=0.01. PLT, platelets; PLT/D, formalin fixed platelets as control

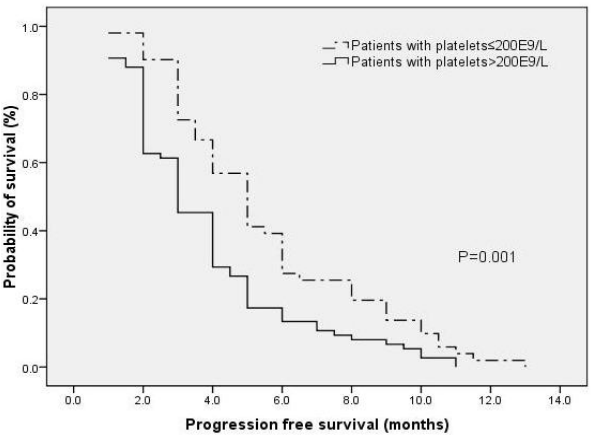

Figure 3. Progression Free Survival in Relation to Platelet Count. Patients with a high platelet count (>200×10^9/L) had a shorter progression free survival [3 months (95% CI: 2.47, 3.53) vs. 5 months (95% CI: 4.14, 5.86), P=0.001]

cell type, TNM stage, and tumor differentiation between patients with high and low platelet counts (Ps>0.05). The median PFS was 3 months (95% CI: 2.47, 3.53) in patients with a high platelet count and 5 months (95% CI: 4.14, 5.86) in those with a low platelet count. The Kaplan Meier PFS curve showed a highly significant separation (P=0.001, Figure 3). In univariate analysis as shown in Table 2, there was a significant association of platelet count with PFS (hazard ratio: 1.70; 95% CI: 1.18, 2.46; P=0.004). In multivariate analysis adjusting for age, gender, cell type, TNM stage and tumor differentiation, the significance remained (hazard ratio: 1.69; 95% CI: 1.16, 2.46; P=0.006, data not shown).

Table 2. Univariate Analysis

Patient characteristics	Unadjusted HR (95% CI)	*P* value
Age (years old)		
≤60	Reference	
>60	1.18 (0.82, 1.68)	0.37
Gender		
Female	Reference	
Male	1.07 (0.75, 1.54)	0.71
Cell type		
Adenocarcinoma	Reference	
Squamous	1.26 (0.78, 2.04)	0.34
TNM stage		
III	Reference	
IV	1.36 (0.77, 2.39)	0.29
Tumor differentiation		
Well	Reference	
Moderately	2.14 (0.86, 5.31)	0.10
Poorly	1.62 (0.62, 4.24)	0.32
Platelet count		
≤200	Reference	
>200	1.70 (1.18, 2.46)	0.004

Discussion

In present study, we showed an elevated platelet count was associated with a shorter PFS in III/IV NSCLC patients. More importantly, our study demonstrated that platelets could enhance lung cancer cell migration and promote the process of NSCLC hematogenous metastasis.

The association between platelets and cancer survival has been recognized. The course of cancer is strongly associated with hypercoagulable state. Our study showed that increased platelet count predicted shorter PFS in advanced NSCLC, which was consistent with previous studies (Tomita et al., 2008; Yu et al., 2013). However, the mechanisms responsible for platelet mediated effect on NSCLC prognosis remained unclear so far. Cho et al. (2012) reported a direct proliferative effect of platelets on ovarian cancer cells. In our study, the lung adenocarcinoma cells showed higher migration rate after incubating with platelets while their proliferation were not affected. The enhancement of cancer cell migration ability is important to promote cancer metastasis. In our mice model, platelets could significantly increase the hematogenous metastasis of lung adenocarcinoma cells. These findings suggested the platelets might affect the NSCLC metastasis by promoting cancer cell migration instead of stimulating proliferation. Different effects of platelets on cancer cells suggest that platelets may have multiple roles in different tumor entities.

The molecular mechanism of platelets affecting cancer metastasis has been extensively studied but remained unclear. Platelets aggregation could prolong the tumor cell survival in the circulation, as a protective thrombus may

shield tumor cells from recognition by the immune system. There was evidence to support the concept that platelets could limit the ability of natural killer (NK) cells to lyse tumor cells in vitro and in vivo (Nieswandt et al., 1999; Palumbo et al., 2005). Platelets may also facilitate tumor cell survival in circulation by increasing the formation of tumor cell clusters that enhance embolization in the microvasculature (Gay et al., 2011). Vascularization within a tumor is a portal through which tumor cells can enter the bloodstream to disseminate. Platelets may stabilize vessel growth during tumor development and thus contribute to this process. Several growth factors such as vascular endothelial growth factor, platelet derived growth factor, and transforming growth factor-β are stored in platelet granules and released on platelet activation. Such platelet-derived factors reportedly induce tumor vascular angiogenesis, growth, and epithelial-to-mesenchymal transition (Italiano et al., 2008; Ho-Tin-Noé et al., 2009). Takagi et al showed the platelet aggregation-inducing factor Aggrus, also known as podoplanin, was frequently upregulated in several types of tumors and enhanced hematogenous metastasis by interacting with and activating the platelet receptor CLEC-2 (Takagi et al., 2013). The development of platelet inhibitors that influence malignancy metastasis represents a promising area of targeted cancer therapy.

In summary, elevated platelet count may indicate a poor prognosis of advanced NSCLC. Platelets might promote the NSCLC hematogenous metastasis process through enhancing cancer cell migration instead of stimulating proliferation. Existing knowledge and further mechanistic studies might suggest platelets and their functions as a new avenue for antimetastatic therapy for NSCLC.

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