Correlation between circulating tumor cells and D-D and platelet in patients with pulmonary malignancies

LIJUN YANG*, HAI DONG*, ZIXUAN LI, YONGYUE PAN, LA QU and ZHIWEN TAN

School of Medicine, Tibet University, Lhasa, Tibet 850000, P.R. China

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Abstract. The aim of the present study was to investigate the correlation between circulating tumor cells (CTC) and D-dimer (D-D) and platelet (PLT) in patients with pulmonary malignancies. A total of 98 patients with lung cancer admitted to West China Hospital, Sichuan University, from June 2016 to February 2017 were enrolled in the present study. D-D and PLT levels were measured in the fasting elbow vein of the patients. The expression of CTC in peripheral blood was detected by negative separation using immunomagnetic beads and immunocytochemical staining. The correlation between CTC and D-D and PLT in patients with lung cancer was analyzed. The mean level of D-D in the peripheral blood of 98 patients was 1.80±1.63 μg/l, and the level of D-D was correlated with distant metastasis (P<0.05). The mean level of PLT in peripheral blood was 305.53±141.22x10^9/l in 98 patients, and the level of PLT was correlated with patient age, clinical stage and distant metastasis (P<0.05). The levels of D-D, PLT and distant metastasis were significantly higher in CTC-positive than in CTC-negative patients (P<0.05). Therefore, CTC can predict the distant metastasis of lung cancer, and the incidence of distant metastasis is high in patients with hypercoagulable state.

Introduction

The mortality rate of lung cancer ranks first among all malignancies and poses a serious threat to human health. The 5-year survival rate is less than 15%, and the incidence is on the increase annually. Most patients develop distant organ metastasis, such as bone, brain, liver, and adrenal gland with progression of the disease, which has a serious impact on the prognosis (1,2).

As one of the necessary pathways for distant metastasis of the tumor is the blood circulation system, tumor cells in the blood circulation system have the same genetic and cytological characteristics as tumor cells in the primary tumor. Therefore, circulating tumor cells (CTC) of lung cancer patients is a major indicator of assessing the patient's condition (3). Platelet (PLT) is an indispensable part of the blood circulation, playing an irreplaceable role in the metastasis of CTC (4). Plasma D-dimer (D-D) is a specific degradation product of cross-linked fibrin, which is a specific marker of secondary fibrinolysis and hypercoagulability *in vivo* (5).

Therefore, in the present study, the correlation between CTC and D-D, and PLT was examined in lung cancer patients, in order to provide a reference for late clinical treatment. The results showed that, CTC is able to predict the distant metastasis of lung cancer, and the incidence of distant metastasis is high in patients with the hypercoagulable state.

Materials and methods

General information. A total of 98 patients with malignant lung cancer admitted to West China Hospital, Sichuan University (Sichuan Sheng, China) from June, 2016 to February, 2017 were selected as subjects of the study, including 51 males and 47 females, aged 48-79 years; mean, 60.63 ± 7.93 years. The present study was approved by the Ethics Committee of Tibet Traditional Medical College (Tibet, China). Signed written informed consent was obtained from the patients and/or guardians.

Inclusion criteria. Inclusion criteria for the study were: i) Patients diagnosed as lung cancer clinically; ii) successful removal of tumor via surgery; iii) without anticoagulant therapy or antitumor treatment; and iv) informed consent was voluntarily signed.

Exclusion criteria. Exclusion criteria for the study were: i) Patients with other malignant tumors; ii) patients with severe heart, liver and kidney dysfunction; iii) patients with infectious diseases or thrombotic disease; and iv) taking hormones or surgical history within 6 months.

CTC-positive diagnostic criteria. CTC-positive diagnostic criteria (6) were determined under a light microscope (Olympus, Tokyo, Japan), and were as follows: i) Integrate cell membrane, Pan-CK staining positive, cytoplasm was brown or dark blue; ii) nuclear cytoplasmic ratio abnormalities; iii) cell diameter greater than 10 μm. Regarding morphology: i) Complete nucleus and cell morphology were observed under

Correspondence to: Dr Lijun Yang, School of Medicine, Tibet University, Jiri Street, Lhasa, Tibet 850000, P.R. China
E-mail: 519420803@qq.com

*Contributed equally

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Table I. D-D level and the correlation with clinicopathological characteristics of patients with lung cancer.

Item	No. of cases (n)	D-D level (μg/l)	t-test	P-value
Age (years)			1.767	0.080
≥60	52	1.76±1.03		
<60	46	1.68±1.06		
Sex			1.617	0.180
Male	51	1.79±1.58		
Female	47	1.81±1.23		
Clinical stage			1.956	0.052
I	12	1.55±0.91		
II	21	1.68±1.16		
III	28	1.39±0.89		
IV	37	1.74±1.32		
Pathologic type			1.926	0.063
Squamous cell carcinoma	44	1.89±0.95		
Adenocarcinoma	33	1.79±1.05		
Small cell carcinoma	21	1.37±0.99		
Distant metastasis			2.253	0.038
No	62	1.35±0.78		
Yes	36	2.45±1.13		

D-D, D-dimer.

light microscope; and ii) the cells were oval, round or long, or long and the diameter was more than 10 μm.

Materials and methods

Instruments and reagents. For D-D detection, an automatic hemagglutination instrument and its supporting reagents (Diagnostica Stago, Gennevilliers, France) were used. For PLT detection, automatic blood cell analyzer and its supporting reagents (Mindray BC6088; Mindray, Shenzhen, China) were employed. Morning fasting elbow vein blood D-D and PLT levels were also measured. The expression of CTC in peripheral blood was detected by negative separation with immunomagnetic beads and immunocytochemical staining.

Methods. The blood and lymphocyte separation medium was placed at room temperature from 18 to 25˚C for 30 min, and 7.5 ml of phosphate buffer and 7.5 ml of anticoagulation was mixed in a 50 ml centrifuge tube, added into a centrifuge tube with 15 ml of lymphocyte separation medium, and centrifuged for 30 min at 3,649 x g at room temperature. The banded plasma mononuclear cells were aspirated and added with 5-fold phosphate buffer, mixed completely, and centrifuged at 2,053 x g for 15 min at room temperature. The supernatant was separated and the lower layer was centrifuged twice.

After the cells were precipitated, they were resuspended in 80 μl of magnetic bead buffer and 20 μl of mouse anti-human CD45 monoclonal antibody (dilution, 1:200; no. 130-098-043; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was added. The antibody was bound to magnetic beads and incubated into 4˚C refrigerator for 5 min. Subsequently, the volume of bead buffer was added 20 times and centrifuged for 10 min at 2,053 x g at room temperature. The supernatant was separated and 500 μl of buffer was added to resuspend the cells. The resuspended cells passed through the MS column and were rinsed twice with 500 μl of the bead buffer to collect the effluent from the column. The cells were again centrifuged at room temperature for 10 min at 2,053 x g, the supernatant was separated, the precipitation was evenly applied to the anti-off slides, air dried at room temperature, and fixed using 4% paraformaldehyde for 10 min. The cells were then washed with 7.5 ml phosphate buffer for 5 min, three times, followed by the addition of anti-spectral cytokeratin (Pan-CK; Cell Signaling Technology, Inc., Danvers, MA, USA), and incubation in a 4˚C refrigerator for 12 h. The cells were then rinsed with 7.5 ml phosphate buffer, and EnVision working solution was added to the rat anti-mouse secondary polyclonal antibody (diilution, 1:800; no. 130-098-105), prior to incubation in a thermostat at 37˚C for 20 min. The cells were washed again with 7.5 ml of phosphate buffer, developed with diaminobenzidine, and then counterstained with hematoxylin for 5 min. Finally, the cells were observed under a light microscope (Mantis Elite, Vision Engineering Ltd.) following steps including differentiation, dehydration, transparent, and neutral gum seal-light.

Observation indicators. The D-D normal range was 0-0.55 μg/l, and the PLT normal range was (125-350)x10^9/l.

Statistical analysis. SPSS 19.0 software (SPSS, Inc., Beijing Xinmeijiahong Technology Co., Ltd.) was used for statistical analysis. Measurement data were determined using the t-test, and countable data were determined using the χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference.

Table II. Relationship between PLT levels and clinicopathological characteristics of lung cancer.

Item	No. of cases (n)	PLT level (x10^9/l)	t-test	P-value
Age (years)			3.376	0.001
≥60	52	367.66±112.46		
<60	46	198.74±108.53		
Sex			1.637	0.110
Male	51	290.55±149.38		
Female	47	293.76±138.71		
Clinical stage			2.580	0.010
I	12	120.46±16.46		
II	21	143.75±18.37		
III	28	261.74±35.89		
IV	37	413.92±61.34		
Pathologic type			1.432	0.303
Squamous cell carcinoma	44	321.47±139.73		
Adenocarcinoma	33	286.83±127.37		
Small cell carcinoma	21	279.64±141.46		
Distant metastasis			2.504	0.022
No	62	215.78±89.43		
Yes	36	446.66±91.57		

PLT, platelet.

Table III. Correlation between lung cancer and CTC and D-D, PLT levels, and distant metastasis.

Groups	No. of cases (n)	D-D level (μg/l)	PLT level (x10^9/l)	Distant metastasis (n, %)
CTC-positive	56	2.39±2.01	342.55±129.47	29 (51.79)
CTC-negative	42	1.04±1.06	236.49±118.95	6 (14.29)
χ^2/t-test		2.801	2.678	8.676
P-value		0.006	0.009	0.004

CTC, circulating tumor cells; D-D, D-dimer; PLT, platelet.

Results

Association between D-D level and clinicopathological characteristics of lung cancer. The average D-D level in the peripheral blood of 98 patients was 1.80±1.63 μg/l, and the level of D-D was correlated with distant metastasis (P<0.05) (Table I).

Association between PLT level and clinicopathological characteristics of lung cancer. The average level of PLT in the peripheral blood of 98 patients was 305.53±141.22x10^9/l. The PLT level was correlated with patient age, clinical stage and distant metastasis (P<0.05) (Table II).

Correlation between CTC and D-D, PLT level and distant metastasis in patients with lung cancer. The incidence of D-D, PLT and distant metastasis in the CTC-positive group was significantly higher than that in the CTC-negative group, and the difference was statistically significant (P<0.05) (Table III).

Discussion

Pulmonary malignant tumor has a high rate of recurrence and distant metastasis, thus the 5-year survival rate is low (7). The hyperplastic and hypercoagulable states of the patients are closely related to the local infiltration and distant metastasis of tumor cells. The tumor cells can affect the coagulation system by secreting inflammatory cytokines, expressing the coagulation protein and the adhesion of normal cells. Additionally, blood vessel endothelial cell injury, blood hypercoagulable state and other factors of malignant tumor patients may lead to distant metastasis (8).

CTC, as a kind of cell in the circulatory system with a high degree of metastasis and high activity, is important in tumor cell metastasis. On the one hand, CTC is a subtype of tumor stem cells. On the other hand, the tumor microenvironment of CTC provides the appropriate conditions for maintaining the survival of tumor cells, and can also provide a metastatic path for tumor cells through complex and extensive intercellular interactions (9). Previous studies have suggested that, in many tumors, D-D and

PLT levels are negatively correlated with the survival of patients and inhibition of PLT activity (10). The reduction of D-D levels is effective for reducing the distant metastasis of malignant tumors, which indicates that PLT and D-D may be involved in maintaining CTC survival and metastatic infiltration.

Therefore, the present study explored the correlation between lung cancer and CTC and D-D and PLT, in order to provide a reference for late clinical treatment.

Tumor cells can secrete cancer coagulation, tissue and other coagulation-related factors, play a role in the coagulation process, activate prothrombin, turning the environment of tumor cells into a hypercoagulable state, thereby forming thrombosis (11). At the same time, the hypercoagulable state can improve the adhesion ability of tumor emboli on the target organ vascular wall, increasing the chance of metastases (12). D-D is a product of cross-linked fibrinolytic decomposition by fibrinolytic enzymes, and serves as an important molecular marker for the diagnosis of fibrinolytic system hyperthyroidism and hypercoagulable state. Moreover, enhanced D-D levels indicate that patients have secondary fibrinolytic system hyperactivity (13). Part of the PLT agglutination activity factor can be secreted by tumor cells, promoting PLT aggregation and adhesion on the surface of tumor cells, releasing PLT-derived growth factor, which can promote tumor cell growth. Thus, the number of PLT and the degree of activation are somewhat correlated with the distant metastasis of tumor cells (14). At the same time, PLT, as a cofactor of the activation of coagulation and tissue factor, can be overexpressed in tumor cells and thus promote PLT aggregation. Thus, activation of PLT can also be combined with fibrin to form tumor cell-fibrin-TCIPA (15). The tumor cells, not only promote PLT aggregation, but also activate PLT through the release of thromboxane A2, adenosine diphosphate and other tumor-associated proteins. Under the effect of activation and aggregation, PLT can form a 'physical barrier' around the CTC to prevent CTC from being damaged in the circulation system (16). The results of the present study have shown that the D-D level in the peripheral blood of 98 patients was 1.80±63 μg/l and D-D was correlated with distant metastasis ($P<0.05$). The level of PLT in the peripheral blood of 98 patients was 305.53±141.22x10^9/l, and the PLT level was correlated with patient age, clinical stage and distant metastasis ($P<0.05$). The levels of D-D, PLT and distant metastasis in CTC-positive patients were significantly higher than those in CTC-negative patients ($P<0.05$). This result also suggests that high levels of D-D and PLT are closely related to postoperative distant metastases in patients with pulmonary malignancies.

In conclusion, CTC is able to predict the distant metastasis of lung cancer, and patients with hypercoagulable state have a higher incidence of distant metastasis. This suggests that in clinical treatment, for some lung cancer patients without contraindications, anticoagulant therapy can be provided, thereby reducing the recurrence and distant metastasis rates of tumor, improving survival and prognosis.

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