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ORIGINAL RESEARCH

Clinical Significance of Red Cell Distribution Width and Circulating Tumor Cells with an Epithelial–Mesenchymal Transition Phenotype in Lung Adenocarcinoma

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Objective: To determine the prognostic value of red cell distribution width (RDW) and circulating tumor cells with epithelial–mesenchymal transition phenotype (M-CTC) in lung adenocarcinoma (LUAD).

Patients and Methods: Clinical and laboratory data of 60 patients with LUAD were collected. CTCs were isolated from their peripheral blood using the CanPatrol^{TM} CTC enrichment method. The indicators of RDW and neutrophil lymphocyte ratio (NLR) were calculated based on the laboratory standards.

Results: A total of 60 LUAD patients were enrolled, of which 19 (31.7%) had high RDW (>0.14) and 32 (53.3%) were positive for M-CTCs. There was no significant correlation between RDW and the clinical characteristics. M-CTC was not significantly associated with tumor size and differentiation, age, gender, tumor stage, and histological type but correlated significantly with lymphatic metastasis (P = 0.044), high NLR (>2.26, P = 0.023), and high RDW (>0.14, P = 0.036). Furthermore, the M-CTC^{+} LUAD patients had a significantly poor recurrence-free survival (RFS; Log rank P =0.001, HR = 2.749, 95% CI = 1.489–5.078) and overall survival (OS; Log rank P =0.022, HR = 2.283, 95% CI = 1.128–4.622) compared to the M-CTC^{-} patients. Similarly, high RDW also correlated with worse RFS (Log rank P = 0.008, HR = 2.331, 95% CI = 1.248–4.353) and OS (Log rank P = 0.004, HR = 0.004, 95% CI = 1.398–5.525).

Conclusion: M-CTC is significantly related to RDW and NLR, and an independent prognostic factor in LUAD.

Keywords: circulating tumor cell, epithelial–mesenchymal, red cell distribution width, lung adenocarcinoma, survival

Introduction

Lung cancer is the leading cause of morbidity and mortality in China and worldwide. Non-small cell lung cancer (NSCLC) accounts for nearly 80% of all lung cancer cases, and includes large cell carcinoma, squamous cell carcinoma and adenocarcinoma, of which lung adenocarcinoma (LUAD) has the highest prevalence of 50%.[1,2] Although the development of novel diagnostic and therapeutic approaches has improved the prognosis of NSCLC patients, the 5-year survival rate of patients with LUAD is only 4–17%,[3,4] mainly due to the lack of simple and effective prognostic biomarkers. Therefore, novel biomarkers need to be identified in order to improve early diagnosis and treatment of LUAD patients.

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Table 1 Patient Characteristics of the Entire Series

Group	n	%
Gender		
Male	30	50
Female	30	50
Age		
≤65	44	73.3
>65	16	16.7
Smoking		
NO	45	75
YES	15	25
Lymphatic metastasis		
N-	28	46.7
N+	32	53.3
Tumor size, cm		
≤4	42	70
>4	18	30
Stage		
I	23	38.3
II	14	23.3
III	17	28.3
IV	6	10
Differentiated degree		
Poorly	30	50
Moderately	24	40
Well	6	10

Studies have established the prognostic relevance of complete blood counts (CBC) in various malignancies, including lung cancer.[5–7] CBC parameters are reliable indices of local and systemic inflammation,[8–10] and cancer patients frequently show significant changes in neutrophil, lymphocyte and platelet counts, red cell distribution width (RDW), systemic immune inflammation index (SII), platelet to lymphocyte ratio (PLR), neutrophil to lymphocyte ratio (NLR) and monocyte to lymphocyte ratio (MLR).[8,11] RDW (%) is a measure of the variability in erythrocyte volume and calculated as the standard deviation of erythrocyte volume/average cell volume × 100. High RDW is associated with the prognosis of liver cancer,[12] breast cancer[13] and gastric cancer,[14] and likely caused by chronic inflammation and poor nutritional status (such as deficiency of iron, folic acid and vitamin B12)[15,16] that frequently accompanies cancer.

The existence of circulating tumor cells (CTCs) was first proposed by Ashworth in 1869.[17] CTCs are epithelial cells that are shed from the primary tumor into circulation and cause tumor metastasis.[18] They are classified into the epithelial (E-CTCs), epithelial–mesenchymal transition (M-CTCs) and mixed (E/M-CTCs) phenotypes.[19] Epithelial–mesenchymal transition (EMT) endows cancer cells with greater invasiveness and is crucial to the process of metastasis.[20–22] Consistent with this, studies show that M-CTCs are closely related to the prognosis and other characteristics of gastric cancer,[23] breast cancer,[24] liver cancer[25] and NSCLC.[26] However, despite millions of tumor cells entering the bloodstream every day, the detection rate of CTCs is very low[27] due to their clearance by

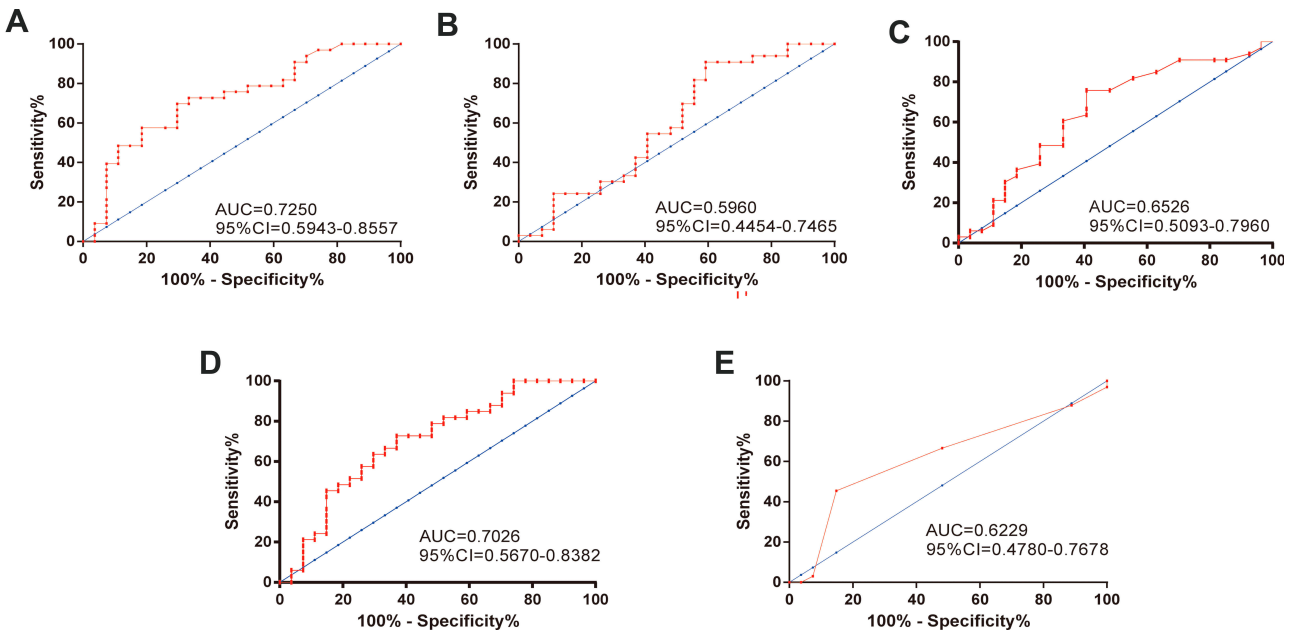

Figure 1 The ROC curves for inflammation index: (**A**) NLR; (**B**) PLR; (**C**) MLR; (**D**) SII and (**E**) RDW.

Table 2 Association Between Patients/Tumor Characteristics with NLR and PLR

Group	n	NLR				PLR			
		NLR≤2.26 N(%)	NLR>2.26 N(%)	P-value	OR(95%CI)	PLR≤108.94 N(%)	PLR>108.94 N(%)	P-value	OR(95%CI)
Gender									
Male	30	11 (36.7)	19 (63.3)	0.073	0.386 (0.136–1.094)	8 (26.7)	22 (73.3)	0.543	0.386 (0.136–1.094)
Female	30	18 (60.0)	12 (40.0)			6 (20.0)	24 (80.0)		
Age									
≤65	44	19 (43.2)	25 (56.8)	0.190	0.459 (0.141–1.476)	9 (20.5)	35 (79.5)	0.385	0.566 (0.156–2.047)
>65	16	10 (62.5)	6 (37.5)			5 (31.3)	11 (68.7)		
Smoking									
NO	45	20 (44.4)	25 (55.6)	0.060	3.437 (0.949–12.445)	12 (26.7)	33 (73.3)	0.301	2.364 (0.464–12.048)
YES	15	9 (60.0)	6 (40.0)			2 (13.3)	13 (86.7)		
Lymphatic metastasis									
N-	28	17 (60.7)	11 (39.3)	0.075	2.576 (0.908–7.308)	7 (25.0)	21 (75.0)	0.775	1.190 (0.359–3.942)
N+	32	12 (37.5)	20 (62.5)			7 (21.9)	25 (78.1)		
Tumor size, cm									
≤4	42	22 (52.4)	20 (47.6)	0.340	1.729 (0.561–5.322)	11 (26.2)	31 (73.8)	0.428	1.774 (0.430–7.323)
>4	18	7 (38.9)	11 (61.1)			3 (16.7)	15 (83.3)		
Stage									
I+II	37	22 (59.5)	15 (40.5)	**0.032**	3.352 (1.111–10.115)	11 (29.7)	26 (70.3)	0.148	2.821 (0.693–11.477)
II+IV	23	7 (30.4)	16 (69.6)			3 (13.0)	20 (87.0)		
Differentiated degree									
Poorly	30	14 (46.7)	16 (53.3)	0.796	0.875 (0.318–2.410)	5 (16.7)	25 (83.3)	0.227	0.467 (0.135–1.609)
Moderately+Well	30	15 (50)	15 (50.0)			9 (30.0)	21 (70.0)		

Note: Bold values indicate statistically significant values.

Abbreviations: CTC, circulating tumor cell; M-CTC, CTCs with epithelial–mesenchymal transition phenotype; OR, risk ratio; CI, confidence interval; NLR, neutrophil lymphocyte ratio; PLR, platelet to lymphocyte ratio.

immune cells or other factors.[28] However, other blood cells like neutrophils and platelets can enhance the survival and distant metastasis of CTCs.[29] For instance, an aberrantly high peripheral blood NLR is significantly correlated to tumor development, since neutrophils secrete vascular endothelial growth factor (VEGF) and proteases that promote CTCs adhesion and seeding in distant organs.[30,31] Lymphocytes on the other hand prevent tumor metastasis by inducing cell death and inhibiting tumor cell proliferation and migration,[28,32] which determines patients' immune response to malignant tumors.[33] Furthermore, the inflammatory response and oxidative stress-induced damage to red blood cells increases RDW, which alters the blood flow[34–36] and may further disseminate the CTCs.

Although the relationship between RDW and tumor prognosis has been established before, the specific role of RDW in LUAD remains to be elucidated. The aim of this study was to explore the correlation between NLR, RDW and M-CTC in LUAD, and determine their respective prognostic values. To this end, we collected clinical and pathological data of 60 LUAD patients, and isolated and typed the peripheral blood CTCs using the advanced CanPatrol^{TM} CTC enrichment technology and in situ hybridization respectively.

Patients and Methods

Study Population and Design

Sixty LUAD patients were enrolled between April 2014 and July 2014 at the First Affiliated Hospital of Guangxi Medical University (Nanning, China) (Supplemnatery Table S1). The inclusion criteria were as follows: (i) pathologically confirmed LUAD, (ii) radical lobectomy and systemic lymph node dissection, (iii) no distant metastasis before surgery, (iv) no history of radiotherapy or chemotherapy before surgery, and (v) availability of complete medical records. The platelet (P), neutrophil (N), monocytes (M) and lymphocyte (L) counts, and the RDW were measured by routine tests in the week before

Table 3 Association Between Patients/Tumor Characteristics with MLR and SII

Group	n	MLR				SII			
		MLR≤0.24 N(%)	MLR>0.24 N(%)	P-value	OR(95%CI)	SII≤491.70 N(%)	SII>491.70 N(%)	P-value	OR(95%CI)
Gender									
Male	30	9 (30.0)	21 (70.0)	0.117	0.429(0.149–1.236)	12 (40.0)	18 (60.0)	0.603	0.762 (0.274–2.121)
Female	30	15 (50.0)	15 (50.0)			14 (46.7)	16 (53.3)		
Age									
≤65	44	15 (34.1)	29 (65.9)	0.127	0.402(0.125–1.294)	16 (36.4)	28 (63.6)	0.076	0.343 (0.105–1.120)
>65	16	9 (56.3)	7 (43.7)			10 (62.5)	6 (37.5)		
Smoking									
NO	45	21 (46.7)	24 (53.3)	0.078	3.500 (0.868–14.110)	21 (46.7)	24 (53.3)	0.370	1.750 (0.515–5.945)
YES	15	3 (20.0)	12 (80.0)			5 (33.3)	10 (66.7)		
Lymphatic metastasis									
N-	28	15 (53.4)	13 (46.6)	**0.048**	2.949 (1.011–8.599)	17 (60.7)	11 (39.3)	**0.013**	3.949 (1.340–11.644)
N+	32	9 (28.1)	23 (71.9)			8 (25.0)	24 (75.0)		
Tumor size, cm									
≤4	42	19 (45.2)	23 (54.8)	0.211	2.148 (0.998–10.262)	22 (52.4)	20 (47.6)	**0.037**	3.850 (1.086–13.647)
>4	18	5 (27.8)	13 (72.2)			4 (22.2)	14 (77.8)		
Stage									
I+II	37	19 (51.4)	18 (48.6)	**0.027**	3.800 (1.165–12.392)	21 (56.8)	16 (43.2)	**0.010**	4.725 (1.444–15.457)
II+IV	23	5 (21.7)	18 (78.3)			5 (21.7)	18 (78.3)		
Differentiated degree									
Poorly	30	10 (30.0)	20 (70.0)	0.294	0.571 (0.201–1.624)	12 (40.0)	18 (60.0)	0.603	0.762 (0.274–2.121)
Moderately+Well	30	14 (46.7)	16 (53.3)			14 (46.7)	16 (53.3)		

Note: Bold values indicate statistically significant values.

Abbreviations: CTC, circulating tumor cell; M-CTC, CTCs with epithelial–mesenchymal transition phenotype; OR, risk ratio; CI, confidence interval; MLR, monocyte-lymphocyte ratio; SII, systemic immune inflammation index.

surgery. SII was calculated as P × N/L, NLR as N/L, MLR as M/L, and PLR as P/L. Five milliliter peripheral blood was collected from patients within three days after surgery into anticoagulant-coated tubes for CTCs isolation or biochemical assays. The study was conducted in accordance with the Declaration of Helsinki. The study was also approved by the ethical committee of the First Affiliated Hospital of Guangxi Medical College, and all patients provided written informed consent.

Isolation of CTCs

The erythrocytes were removed from the peripheral blood samples using the erythrocyte lysis buffer, and the plasma was filtered through an 8μm pore size filter membrane using the CanPatrol^{TM}[37,38] immune capture and nanofiltration-based CTC enrichment system. The isolated CTCs were then typed for CD45 and the EMT markers (EpCAM and vimentin) using RNA in situ hybridization (ISH).[37]

RNA ISH

The CTCs were digested with protease (Qiagen GmbH, Hilden, Germany) and hybridized with EpCAM, vimentin and CD45 probes (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 42°C for 2 hours. After washing thrice with 1 mL washing buffer to remove unbound probes, the cells were incubated with preamplifier solution at 42°C for 20 minutes, cooled, washed again, and incubated with the amplifier solution at room temperature for 1h. The cells were then incubated with Alexa Fluor 594-vimentin, Alexa Fluor 488-EpCAM and Alexa Fluor 647-CD45 at 42°C for 20 minutes, washed, and counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 minutes at room temperature. The stained cells were observed under a fluorescence microscope (Olympus Corporation, Tokyo, Japan), and the $EpCAM^+$ $vimentin^-$ (E-CTCs), $EpCAM^+$ $vimentin^+$ (biphenotypic E/MCTCs) and $EpCAM^-$ $vimentin^+$ (M-CTCs) phenotypes were identified.

Table 4 Association Between Patients/Tumor Characteristics with RDW

Group	n	RDW			
		RDW≤0.14 N(%)	RDW>0.14 N(%)	P-value	OR(95%CI)
Gender					
Male	30	18 (60.0)	12 (40.0)	0.169	0.457(0.149–1.396)
Female	30	13 (43.3)	7 (56.4)		
Age					
≤65	44	28 (63.6)	16 (36.4)	0.204	0.404 (0.100–1.634)
>65	16	13 (81.2)	3 (18.8)		
Smoking					
NO	45	33 (73.3)	12 (26.7)	0.155	2.406 (0.717–8.074)
YES	15	8 (53.3)	7 (46.7)		
Lymphatic metastasis					
N-	28	23 (82.1)	5 (17.9)	0.302	1.800 (0.590–5.491)
N+	32	18 (56.3)	14 (43.7)		
Tumor size, cm					
≤4	42	32 (76.2)	10 (23.8)	0.050	3.200 (0.998–10.262)
>4	18	9 (50.0)	9 (50.0)		
Stage					
I+II	37	27 (73.0)	10 (27.0)	0.329	1.736 (0.573–5.256)
II+IV	23	14 (60.9)	9 (39.1)		
Differentiated degree					
Poorly	30	17 (56.7)	13 (43.3)	0.057	0.327 (0.104–1.032)
Moderately+Well	30	24 (60.0)	6 (40.0)		

Abbreviations: CTC, circulating tumor cell; M-CTC, CTCs with epithelial–mesenchymal transition phenotype; OR, risk ratio; CI, confidence interval; RDW, red cell distribution width.

Follow-Up

All patients were followed up through outpatient review or telephone interviews till July 30, 2019. Recurrence-free survival (RFS) was defined as the date from surgery to disease recurrence or the last follow-up. Overall survival (OS) was defined as the time from surgery to death for any reason or the last recorded follow-up visit.

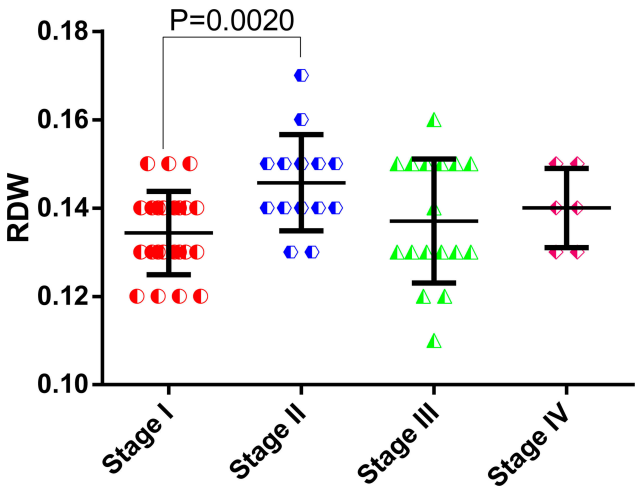

Figure 2 Distribution of RDW in LUAD patients according to tumor stage.

Statistical Analysis

All statistical analysis was performed using SPSS version 19.0 (SPSS Inc., Chicago, Illinois, USA) and the graphs were drawn using GraphPad Prism version 5.0 (GraphPad software, Inc., La Jolla, CA, USA). Time-dependent receiver operating characteristic (ROC) curves were plotted in order to establish the cutoffs for low and high NLR, PLR, MLR, SII and RDW relative to the respective baseline values, optimal sensitivity, specificity, and area under the curve (AUC) for prediction of death from all causes. Kaplan–Meier survival curves were plotted to determine RFS and OS of patients demarcated on the basis of M-CTC, NLR, PLR, MLR, SII and RDW. The hazard rates (HRs) and 95% confidence intervals (CI) were calculated by univariate and multivariate Cox

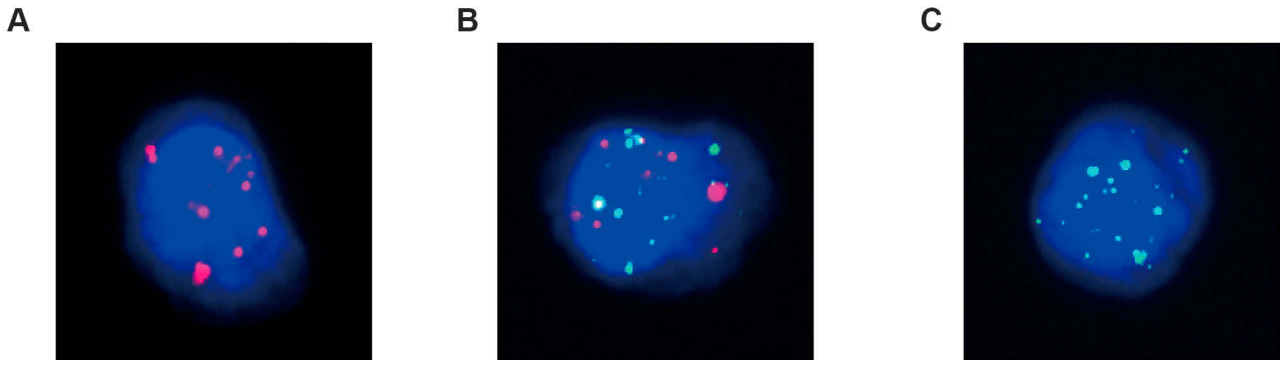

Figure 3 Representative images showing CTCs phenotypes. (**A**) E-CTC; (**B**) E/M-CTC and (**C**) M-CTC. Red CK19 and green – Twist. Magnification – 100×.

proportional hazard regression model. P values less than 0.05 were considered statistically significant.

Results

A total of 60 patients with LUAD were enrolled from April 2014 to July 2014, and their characteristics are summarized in Table 1. There were 30 male and female patients each (50%), and their median age was 59 years (33–79 years, 59.68 ± 9.16 years). Forty-four patients were younger than 65 years and 16 were older than 65 years. In addition, 12 patients (25%) had a history of smoking and 45 (75%) were non-smokers. In terms of oncological parameters, 28 patients (46.7%) had no lymphatic metastasis and 32 (53.3%) presented with lymphatic metastasis, while 18 (30%) and 42 (70%) patients had primary tumors > 4 cm and ≤ 4 cm respectively. Furthermore, 23 (38.3%), 14 (23.3%), 17 (28.3%) and 6 (10%) patients were respectively at stage I, II, III and IV, resulting in 37 (61.7%) patients at early stage (I + II) and 23 (38.3%) at the advanced stage (III + IV). Finally, 30 (50%) patients had poorly differentiated tumors, 24 (40%) moderately differentiated tumors and 6 (10%) presented highly differentiated tumors.

The average NLR in patient peripheral blood is 3.329 ± 3.877 (1.11–29.27). According to the ROC curve, the cut-off value, sensitivity, specificity and area under the curve (AUC) for NLR in our cohort were respectively 2.26, 69.7%, 70.4% and 0.7250 (95% CI=0.5943–0.8557) (Figure 1A). The patients were divided into the NLR ≤ 2.26 (31, 51.7%) and NLR > 2.26 (29, 48.3%) groups, and as shown in Table 2, NLR was significantly correlated with the staging (P = 0.0032, OR = 3.352, 95% CI = 1.111–10.115) but not with other clinical characteristics. The average value of PLR is 157.87 ± 79.28 (44.90–398.99), and its cut-off value in the current study was 108.94 (Figure 1B). The ROC curve also indicated that the sensitivity and specificity were 90.9–40.7% respectively, and the AUC was 0.5960 (95% CI = 0.4454–0.7465). There were 46 (76.7%) patients with high PLR and 14 (23.3%) with low PLR. The relationship between PLR and clinical characteristics are summarized in Table 2, which indicate no significant correlation. The cut-off value of MLR was calculated to be 0.245 (Figure 1C) compared to its average value of 0.347 ± 0.03 (0.10–1.49). The sensitivity and specificity were 75.8–59.3% respectively, and the AUC was 0.6526 (95% CI = 0.5093–0.7960). There were 30 (50%) patients with MLR> 0.245 and 30 (50%) with MLR≤0.245. As shown

Table 5 Positive Expression Rate of CTCs in Each LUAD Stage n (%)

Stating	Numbers	CTCs	E/M-CTC	E-CTC	M-CTC	Median CTCs	CTCs Average	CTCs Range
I	23	22 (95.7)	20 (57.0)	13 (56.5)	8 (34.8)	5	8.9	0–54
II	14	13 (92.9)	10 (71.4)	10 (71.4)	8 (57.1)	4.5	10.4	0–68
III	17	16 (94.1)	16 (94.1)	7 (41.2)	11 (64.7)	6	9.8	0–43
IV	6	6 (100)	4 (66.7)	2 (33.3)	5 (83.3)	6.5	8.8	1–20
Total	60	57 (95.0)	40 (66.7)	32 (53.3)	32 (53.3)	5	9.5	0–68

Abbreviations: CTC, circulating tumor cell; M-CTC, CTCs with epithelial–mesenchymal transition phenotype; E-CTC, CTCs with epithelial phenotype; M-CTC, CTCs with epithelial–mesenchymal transition phenotype; E/MCTCs, CTCs with biphenotypic phenotype.

Table 6 Association Between Patients/Tumor Characteristics with M-CTC

Group	n	M-CTC			
		M-CTC (-) N(%)	M-CTC (+) N(%)	P-value	OR(95%CI)
Gender					
Male	30	13 (43.3)	17 (56.7)	0.605	0.765 (0.277–2.114)
Female	30	15 (50.0)	15 (50.0)		
Age					
≤65	44	20 (45.5)	24 (54.5)	0.755	0.833 (0.265–2.620)
>65	16	8 (50.0)	8 (50.0)		
Smoking					
NO	45	23 (51.1)	22 (48.9)	0.237	2.091 (0.6116–7.099)
YES	15	5 (33.3)	10 (66.7)		
Lymphatic metastasis					
N-	28	17 (60.7)	11 (39.3)	**0.044**	2.950 (1.030–8.451)
N+	32	11 (34.4)	21 (65.6)		
Tumor size, cm					
≤4	42	21 (50.0)	21 (50.0)	0.431	1.571 (0.511–4.837)
>4	18	7 (38.9)	11 (61.1)		
Stage					
I+II	37	20 (54.1)	17 (45.9)	0.149	2.206 (0.753–6.459)
II+IV	23	8 (34.8)	15 (65.2)		
Differentiated degree					
Poorly	30	13 (43.3)	17 (56.7)	0.605	0.765 (0.277–2.114)
Moderately+Well	30	15 (50.0)	15 (50.0)		
NLR					
≤2.26	29	18 (62.1)	11 (37.9)	**0.023**	3.436 (1.187–9.947)
>2.26	31	10 (32.3)	21 (67.7)		
PLR					
≤108.94	13	8 (61.5)	5 (38.5)	0.138	2.558 (0.740–8.846)
>108.94	47	20 (42.6)	27 (57.4)		
MLR					
≤0.24	24	13 (54.2)	11 (45.8)	0.343	1.655 (0.584–4.686)
>0.24	36	15 (31.9)	21 (78.1)		
RDW					
≤0.14	41	21 (51.2)	20 (48.8)	**0.036**	3.578 (1.085–11.795)
>0.14	19	7 (36.8)	12 (63.2)		
SII					
≤491.70	23	13 (56.5)	10 (43.5)	0.331	1.667 (0.595–4.669)
>491.70	37	15 (40.5)	22 (59.5)		

Note: Bold values indicate statistically significant values.

Abbreviations: CTC, circulating tumor cell; M-CTC, CTCs with epithelial–mesenchymal transition phenotype; OR, risk ratio; CI, confidence interval; NLR, neutrophil lymphocyte ratio; PLR, platelet to lymphocyte ratio; MLR, monocyte-lymphocyte ratio; SII, systemic immune inflammation index; RDW, red cell distribution width.

in Table 3, MLR was significantly correlated with lymphatic metastasis (P = 0.048, OR = 2.949, 95% CI = 1.011–8.599) and tumor stage (P = 0.027, OR = 3.800, 95% CI = 1.165–12.392).

The average value of SII is 837.01 ± 851.80 (113.60–5790.15), and its cut-off value was 491.75 in the current study (Figure 1D). The ROC curve indicated that the sensitivity, specificity and AUC were respectively 72.7%, 63%

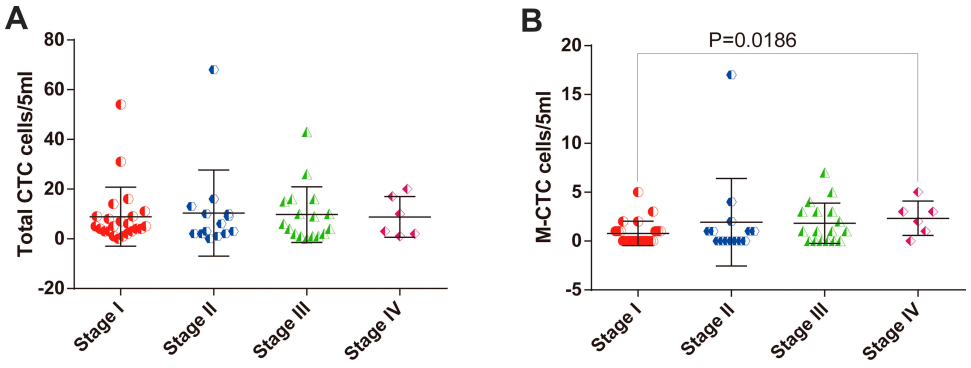

Figure 4 Distribution of CTC and M-CTC counts in LUAD patients according to tumor stage. (**A**) Total CTCs and (**B**) M-CTC.

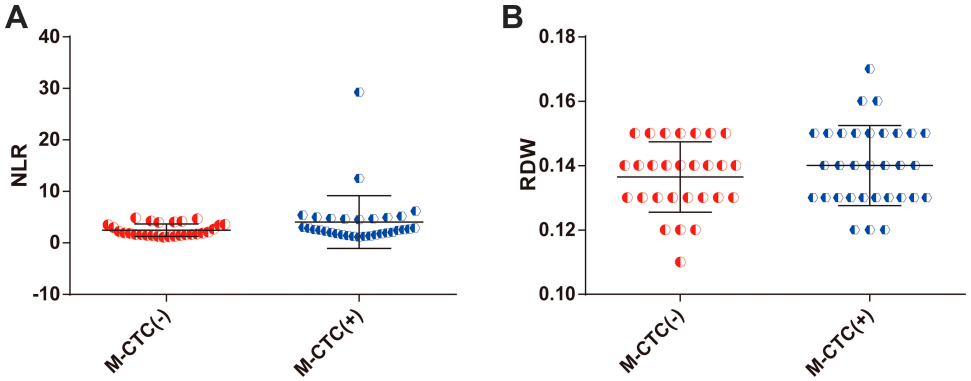

Figure 5 NLR and RDW in M-CTC^{+} and M-CTC^{-} patients. (**A**) NLR and (**B**) RDW.

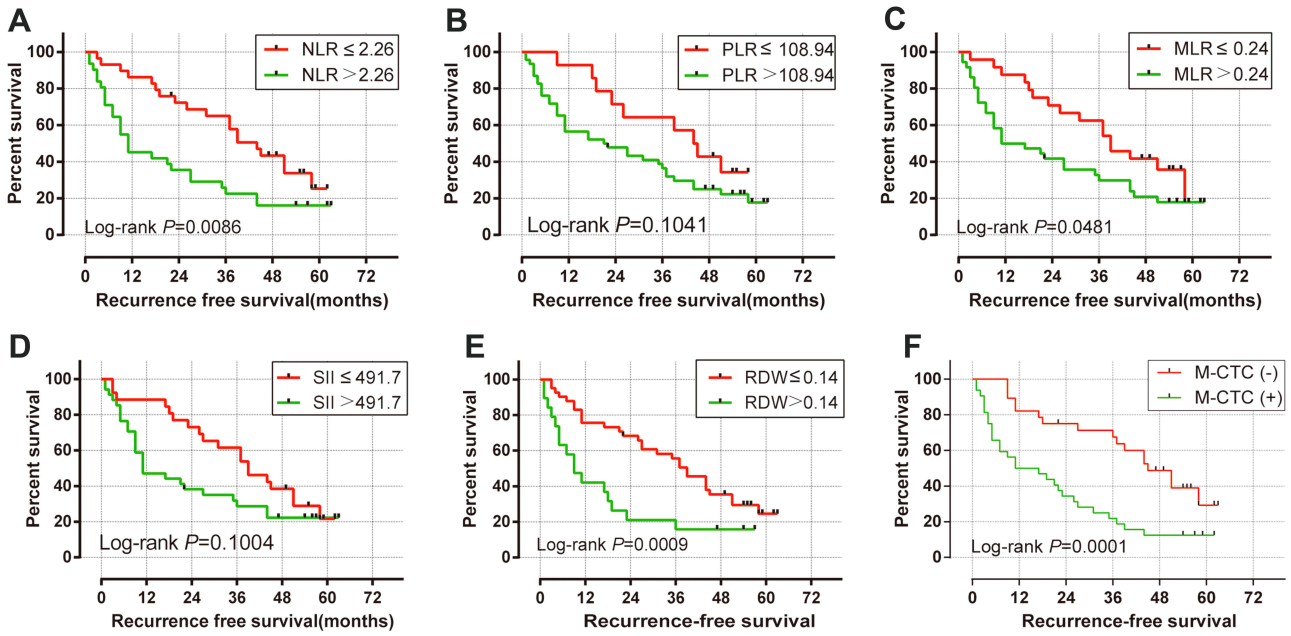

Figure 6 Kaplan–Meier curve of RFS in LUAD patients: (**A**) NLR; (**B**) PLR; (**C**) MLR; (**D**) SII; (**E**) RDW and (**F**) M-CTC.

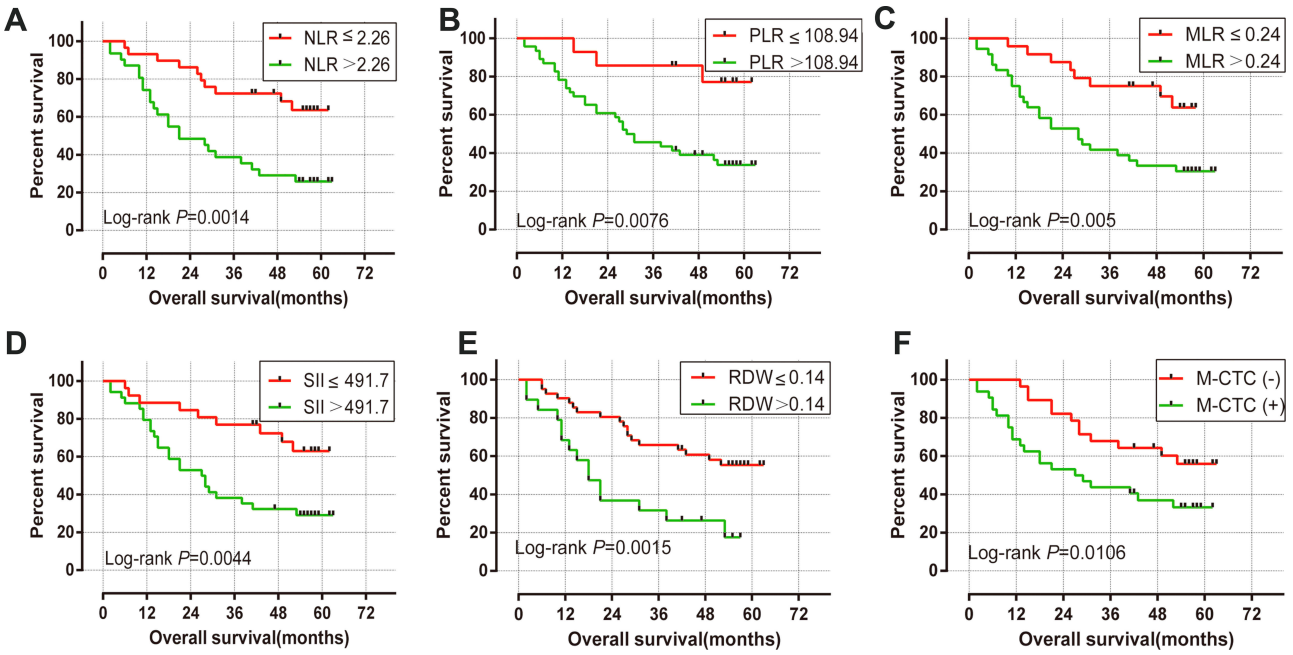

Figure 7 Kaplan–Meier curve of OS in LUAD patients: (**A**) NLR; (**B**) PLR; (**C**) MLR; (**D**) SII; (**E**) RDW and (**F**) M-CT.

and 0.7026 (95% CI = 0.5670–0.8382). Accordingly, 34 (56.7%) and 26 (43.3%) patients were divided into the SII> 491.75 and SII≤491.75 groups respectively. As shown in Table 3, SII was significantly correlated to lymphatic metastasis (P = 0.013, OR = 3.949, 95% CI = 1.340–11.644), tumor size (P = 0.037, OR = 3.850, 95% CI = 1.086–13.647) and stage (P = 0.010, OR = 4.725, 95% CI = 1.444–15.457). The average value of RDW is 0.1383 ± 0.0118 (0.11–0.17), and its cut-off was determined to be 0.14 from the ROC curve (Figure 1E). The sensitivity and specificity of RDW were 45.9–85.2% respectively, and the AUC was 0.6229 (95% CI = 0.4780–0.7678). Nineteen patients showed RDW>0.14 and 41 had RDW≤0.14. Although RDW was not significantly associated with the clinical characteristics (Table 4), it increased with stage progression (Figure 2) and showed statistical significance with stage I and II (P = 0.0020).

Table 7 Univariate and Multivariate Statistical Analyses of Recurrence-Free Survival

Variable	Level	Univariate		Multivariate	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Gender	Women/Men	0.547(0.302–0.992)	**0.047**	0.507 (0.230–1.118)	0.092
Age	≤65/>65	0.845(0.428–1.670)	0.629		
Smoking	Yes/No	2.076(1.083–3.977)	**0.028**	0.814 (0.354–1.868)	0.626
M-CTC	Yes/Not	2.749(1.489–5.078)	**0.001**	2.818 (1.431–5.531)	**0.003**
Lymphatic metastasis	N0/N+	2.316(1.266–4.283)	**0.006**	0.991 (0.356–2.760)	0.987
Tumor size, cm	≤4/>4	2.562(1.378–4.763)	**0.003**	1.682 (0.739–3.828)	0.215
Stage	I+II/III+IV	1.873(1.026–3.420)	**0.041**	1.918 (0.709–5.184)	0.199
Differentiated degree	Poorly/Moderately +Well	0.412(0.225–0.756)	**0.004**	0.517 (0.243–1.101)	0.087
NLR	≤2.26/>2.26	2.158(1.187–3.923)	**0.012**	1.451 (0.678–3.105)	0.338
PLR	≤108.94/>108.94	1.801(0.865–3.751)	0.116		
MLR	≤0.24/>0.24	1.821(0.985–3.365)	0.056		
SII	≤491.70/>491.70	1.627(0.895–2.957)	0.110		
RDW	≤0.14/>0.14	2.331(1.248–4.353)	**0.008**	1.981 (0.953–4.122)	0.067

Note: Bold values indicate statistically significant values.

Abbreviations: CTC, circulating tumor cell; M-CTC, CTCs with epithelial–mesenchymal transition phenotype; OR, risk ratio; CI, confidence interval; NLR, neutrophil lymphocyte ratio; PLR, platelet to lymphocyte ratio; MLR, monocyte-lymphocyte ratio; SII, systemic immune inflammation index; RDW, red cell distribution width; HR, hazard ratio; RFS, recurrence-free survival.

Table 8 Univariate and Multivariate Statistical Analyses of Overall Survival

Variables	Level	Univariate		Multivariate	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Gender	Women/Men	0.609(0.309–1.201)	0.152		
Age	≤65/>65	1.044(0.487–2.238)	0.912		
Smoking	Yes/No	2.711(1.334–5.511)	**0.006**	1.537 (0.635–3.715)	0.340
M-CTC	Yes/Not	2.283(1.128–4.622)	**0.022**	2.490 (1.141–5.431)	**0.022**
Lymphatic metastasis	N0/N+	1.847(0.930–3.668)	0.080		
Tumor size, cm	≤4/>4	2.349(1.179–4.681)	**0.015**	1.366 (0.572–3.264)	0.483
Stage	I+II/III+IV	2.746(1.394–5.409)	**0.003**	2.452 (1.040–5.782)	**0.040**
Differentiated degree	Poorly/Moderately +Well	0.366(0.180–0.746)	**0.006**	0.611 (0.259–1.442)	0.261
NLR	≤2.26/>2.26	2.879(1.398–5.930)	**0.004**	1.171 (0.294–4.664)	0.823
PLR	≤108.94/>108.94	3.299 (1.159–9.389)	**0.025**	2.459 (0.614–9.855)	0.204
MLR	≤0.24/>0.24	2.649 (1.233–5.689)	**0.013**	0.855 (0.210–3.476)	0.827
SII	≤491.70/>491.70	2.635 (1.254–5.539)	**0.011**	1.436 (0.437–4.712)	0.551
RDW	≤0.14/>0.14	2.779 (1.398–5.525)	**0.004**	2.508 (1.084–5.804)	**0.032**

Note: Bold values indicate statistically significant values.

Abbreviations: CTC, circulating tumor cell; M-CTC, CTCs with epithelial–mesenchymal transition phenotype; OR, risk ratio; CI, confidence interval; NLR, neutrophil lymphocyte ratio; PLR, platelet to lymphocyte ratio; MLR, monocyte-lymphocyte ratio; SII, systemic immune inflammation index; RDW, red cell distribution width; HR, hazard ratio; CI, confidence interval; OS, overall survival.

The distribution of CTC phenotypes among the 60 LUAD patients is shown in Figure 3A–C. The positive rate of CTCs was 95% (0 to 68), and the median and average values were 5 and 9.5 ± 12.6 respectively (Table 5). The CTC load increased with disease progression, but did not reach statistical significance (Figure 4A). The positive rates of M-CTC increased steadily to 34.8%, 57.1%, 64.7–83.3% at stages I, II, III and IV respectively (P = 0.0186 between stage I and IV, Figure 4B), while that of CTCs and E-CTCs were unaffected by LUAD progression. Furthermore, the M-CTC^{+} patients had significantly higher risk of lymphatic metastasis (P = 0.044, OR = 2.950, 95% CI = 1.0-0-8.451), RDW (P = 0.036, OR = 3.578, 95% CI = 1.085–11.795) and NLR (P = 0.023, OR = 3.436, 95% CI = 1.187–9.947) compared to the M-CTC^{-} patients (Table 6, Figure 5A and B).

All patients were followed for at least 60 months, during which period 33 (55%) died and 45 (75%) experienced recurrence. NLR, PLR, MLR, SII, LDW and M-CTC levels had different impacts on RFS (Figure 6A–F) and OS (Figure 7A–F). High NLR was associated with significantly worse RFS (P = 0.0086) and OS (P = 0.0014), whereas patients with low PLR had better OS compared to those with high PLR (P=0.0076), although RFS (P=0.104) was not significantly affected. In addition, high MLR showed a significant correlation with worse RFS (P = 0.0481) and OS (P = 0.005). Patients with low SII had markedly better OS compared to patients with high SII (P = 0.0044), while RFS was not significantly affected by this parameter (P = 0.1004). Low RDW also correlated to favorable RFS (P = 0.0009) and OS (P = 0.0015), and consistent with this, the M-CTC^{-} patients showed both better RFS (P = 0.0001) and OS (P = 0.0106).

Univariate analysis showed that gender (P = 0.047, HR = 0.547, 95% CI = 0.302–0.992), smoking (P = 028, HR = 2.076, 95% CI = 1.083–3.977), M-CTC (P = 0.001, HR = 2.749, 95% CI = 1.489–5.078), lymphatic metastasis (P = 0.006, HR = 2.316, 95% CI = 1.266–4.283), tumor size (P = 0.003, HR = 2.562, 95% CI = 1.378 −4.763), stage (P = 0.041, HR = 1.873, 95% CI = 1.026–3.420), degree of differentiation (P = 0.004, HR = 0.412, 95% CI = 0.225–0.756) and RDW (P = 0.008, HR = 2.331, 95% CI = 1.248–4.353) were significantly associated with RFS (Table 7), of which M-CTC was an independent factor of recurrence as per multivariate analysis (P = 0.003, HR = 2.818, 95% CI = 1.431–5.531). Likewise, smoking (P = 0.006, HR = 2.711, 95% CI = 1.334–5.511), M-CTC (P = 0.022, HR2.283, 95% CI = 1.128–4.622), tumor size (P = 0.015, HR = 2.349, 95% CI = 1.179–4.681), staging (P = 0.003, HR = 2.746, 95% CI = 1.394–5.409), degree of differentiation (P = 0.006, HR = 0.366, 95% CI = 0.180–0.746), NLR (P = 0.004, HR = 2.879, 95% CI = 1.398–5.930), MLR (P = 0.013, HR = 2.649, 95% CI = 1.233–5.689), PLR (P = 0.025, HR = 3.299, 95% CI = 1.159–9.389), SII (P = 0.011, HR = 2.635, 95% CI = 1.254–5.539) and RDW (P = 0.004, HR = 2.779, 95% CI = 1.398–5.525) were significantly correlated

with OS, and M-CTC (P = 0.022, HR = 2.490, 95% CI = 1.141–5.431), stage (P = 0.040, HR = 2.452, 95% CI = 1.040–5.782) and RDW (P = 0.032, HR = 2.508, 95% CI = 1.084–5.804) were the independent factors (Table 8).

Discussion

This study is the first to explore the relationship between RDW and M-CTC, and determine their prognostic relevance in LUAD. RDW is a widely available by the vast majority of automated analysis. Reflecting the size heterogeneity of the circuiting erythrocytes, higher RDW values are suggestive of increased variation of red cell volumes (anisocytosis). We found that patients with higher RDW and M-CTC load had worse prognosis, and both increased with tumor progression. In addition, RDW was also determined to an independent risk factor, although the underlying mechanism through which RDW affects prognosis is still unclear. Tumor progression frequently triggers an inflammatory response that further exacerbates tumor growth, invasion and angiogenesis, and eventually promotes metastases.[29,32,39] Inflammation also lowers red blood cell survival by destroying their membranes, leading to increased RDW and red blood cell atypia, thereby altering blood flow through microcirculation and likely promoting M-CTC dispersion.[34] However, further research is needed to elucidate the relationship between RDW, inflammation and tumor metastasis.

CTCs are closely associated with distant metastasis in various malignancies. We found that both CTC and M-CTC counts increased with tumor progression, and patients with lymphatic metastasis had higher M-CTC positive rates. M-CTCs are regarded as the most malignant CTC. Therefore, patients with positive of M-CTC have a greater chance of early recurrence. Current research also confirms this. Metastasis involves EMT of tumor cells that results in the loss of cell-to-cell contact and cellular polarity, along with degradation of the extracellular matrix and basement membrane, which increase tumor cell migration and invasion into adjacent tissues.[40,41] In line with this, both RFS and PFS were significantly worse in the M-CTC$^+$ LUAD patients, and M-CTC was also an independent factor of worse prognosis.

NLR and RDW are established risk factors in multiple malignancies, and the M-CTC count was positively correlated with both factors in the LUAD patients in agreement with the findings of Wu et al.[42] Studies show that neutrophils secrete vascular endothelial growth factor (VEGF) and proteases into circulation, which promote CTCs adhesion and seeding in distant organs.[30,31] Lymphocytes on the other hand inhibit tumor metastasis by inducing cell death and[28,32] mediating an immune response against the malignant tumors.[33] Furthermore, inflammation and oxidative stress-induced damage to red blood cells increases RDW and alters microcirculation,[34–36] which further promote CTC metastasis.

There are certain limitations to our study. For instance, the study was retrospective in nature and performed at a single center on a small number of patients. In addition, we did not elucidate the relationship between M-CTC, RDW and NLR. Our findings need to be validated in multicenter prospective studies on larger cohorts. Nevertheless, we showed for the first time that RDW is associated with M-CTC and LUAD prognosis.

Conclusion

RDW and M-CTC are independent predictors of prognosis in patients with LUAD, and RDW is an economical and convenient prognostic biomarker for LUAD.

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Disclosure

Huajian Peng and Xiang Tan are co-first authors. The authors declare that they have no competing interests.

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