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Identify the Clinicopathological Characteristics of Lung Carcinoma Patients Being False Negative in Folate Receptor Based Circulating Tumor Cell Detection

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In lung cancer diagnosis, folate receptor (FR)–based circulating tumor cell (CTC) has shown its ability to distinguish malignancy from benign disease to some extent. However, there are still some patients that cannot be identified by FR-based CTC detection. And studies comparing the characteristics between true positive (TP) and false negative (FN) patients are few. Thus, the study comprehensively analyzes the clinicopathological characteristics of FN and TP patients in the current study. According to inclusion and exclusion criteria, 3420 patients are enrolled. Combining the pathological diagnosis with CTC results, patients are divided into FN and TP groups, and clinicopathological characteristics are compared between two groups. Compared with TP patients, FN patients have smaller tumor, early T stage, early pathological stage, and without lymph node metastasis. Epidermal growth factor receptor (EGFR) mutation status is different between FN and TP group. And this result is also demonstrated in lung adenocarcinoma subgroup but not in lung squamous cell carcinoma subgroup. Tumor size, T stage, pathological stage, lymph node metastasis, and *EGFR* mutation status may influence the accuracy of FR-based CTC detection in lung cancer. However, further prospective studies are needed to confirm the findings.

1. Introduction

Lung cancer is still one of the cancers with high mortality and morbidity. In the Surveillance, Epidemiology, and End Results Program (SEER) database and the National Center for Health Statistics, there were 236740 new cases of lung cancer and 130180 deaths due to lung cancer in the year of 2020,[1] accounting for 11.4% of new cancer incidences and 18.0% of cancer deaths, respectively.[2] Because the disease usually lacks specific clinical symptoms at early stage, the majority of lung cancers are diagnosed at advanced stage. The 5 year survival rate for stage IA can exceed 90%, while for patients at stage IV it can be <10%,[3] which underscored the importance of early diagnosis and intervention.

Circulating tumor cells (CTCs) were first observed in a metastatic cancer patient in 1869. These cells would be released into blood stream during the formation, growth, and metastasis of cancer.[4] As CTCs derived from primary or metastatic sites of tumor, CTC examination would be a non-invasive approach to get a snapshot of cancer. However, CTC is rare in blood of metastatic cancer patients, as only one cell in per 10^9 blood cells, which makes the capture of CTC a certain amount of challenge.[5] With the development of isolation technology, many approaches based on physical characteristics or surface markers of CTC have been applied in CTC isolation.[6]

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Currently, CTCs have been isolated in peripheral blood of cancer patients such as metastatic prostate cancer, breast cancer, lung cancer[7] and have become a crucial source for liquid biopsy to be used in diagnosing cancer at early time, evaluating the risk of relapse or metastasis, selecting appropriate therapy, monitoring the effect of therapy, exploring mechanisms of drug resistance, and so on.[8]

CTCs are detected not only in peripheral blood but also in pulmonary vein, even at early stage lung cancer.[7c,9] Meanwhile, CTC detection based on different characteristics have also been used in lung cancer diagnosis. A prospective study using a physical based CTC isolation technique, the isolation by size of epithelial tumor cell technique (ISET), reported that the sensitivity of CTC in screening lung cancer was only 26.3% and suggested ISET-based CTC detection could not predict lung cancer or extrapulmonary cancer progression.[10] Kanayama et al. using epithelial cell adhesion molecules (EpCAM) based positive enrichment reported that CTCs were captured in 47.2% lung cancer patients.[11] However, epithelial-to-mesenchymal transition (EMT) was common in cancer progression and cancer cells would lose their epithelial marker in this process that would cause false negative of EpCAM-based CTC detection technique.[12] Thus, another study using both EpCAM and mesenchymal marker demonstrated that the sensitivity of lung cancer diagnosis could be reach to 81.6%.[13] As folate receptor (FR) was overexpressed on cancer cells,[14] FR-based CTC detection was also established,[15] and the sensitivity of this approach in diagnosing lung cancer was range from 74.40% to 87.05%.[15b-d] However, it is still inevitable that some lung cancer patients are not be detected by FR-based CTC examination, and reports that comprehensively analyzing the characteristics of these patients are still lack.

In this research, we focused on lung cancer of epithelial origin and comprehensively compared the characteristics of lung cancer patients between true positive and false negative in FR-based CTC detection. And we also comprehensively analyzed the correlation between CTC result and clinicopathological characteristics in lung cancer, through that we might find out the relevant factors affecting the accuracy of CTC detection.

2. Results

2.1. Characteristics of Participants

In this study, we totally collected 3573 patients who underwent surgery because of persistent imaging abnormalities of chest computed tomography (CT). According to inclusion and exclusion criteria, 18 patients were excluded for having no clear pathological diagnosis, five were excluded due to the paradoxical information in pathological records, 33 were not primary lung tumor, 49 were not have proper CTC test, and 48 were not epithelial derived lung cancer. After selection, 3420 patients met the criteria and were enrolled for further analysis (**Figure 1**). According to the cutoff value of CTC, 8.70 folate unit (FU) per 3 mL blood, 2470 patients (72.2%) were defined as CTC positive, and 950 patients (27.8%) were defined as CTC negative. In these patients, 3045 patients (89.0%) were diagnosed as malignant disease, and 375 (11.0%) patients were diagnosed as benign disease, including inflammation, fibrosis, tuberculosis, pulmonary cryptococcosis, bronchiectasis, precancerous lesion, and so on, by post-operation pathology. The age of patients with non-malignant disease was 55 (47, 63) years old and 198 patients (52.8%) were female. The age of patients with lung carcinoma was 58 (49, 56) years old, and 1831 patients (60.1%) were female. Among them, 819 patients (26.9%) were at stage 0, 1907 patients (62.6%) were at stage I, 149 patients (4.9%) were at stage II, 156 patients (5.1%) were at stage III and 14 patients (0.5%) were at stage IV. The reason why stage IV patients underwent surgery was that the pre-operation examination showed no metastatic sign, while the post-operation pathological examination reported pleural metastasis.

2.2. Comparing Clinicopathological Features between True Positive and False Negative Patients

2206 patients were confirmed as true positive (TP) group, and 839 patients were false negative (FN) group. Thus, the sensitivity of this method in detecting epithelial lung cancer was 72.4%. By comparing TP group and FN group, we found that FN group tended to have more young patients (58.2% vs 53.5%, $P = 0.021$). The FN group had relatively more lung adenocarcinoma (LUAD) patients and less lung squamous cell carcinoma (LUSC) patients ($P = 0.014$). Meanwhile, FN group had more patients with tumor no larger than 3 cm ($P_{FDR} = 0.027$ ≤3 cm vs >5 cm, $P_{FDR} = 0.027$ ≤3 cm vs 3–5 cm) and the examination of CTC tended to be negative in patients with lower T stage ($P_{FDR} = 0.039$ Tis vs T3-4, $P_{FDR} = 0.039$ T1-2 vs T3-4). But we did not find any difference of pleura infiltration between TP and FN group ($P = 0.112$). For N stage, FN group had less patients having lymph node metastasis (5.4% vs 7.9%, $P = 0.014$). For pathological stage, we also found the composition of pathological stage was different between TP and FN group ($P = 0.019$) and patients with advanced stage lung carcinoma tended to be CTC positive ($P_{FDR} = 0.013$ 0 vs III-IV, $P_{FDR} = 0.013$ I-II vs III-IV). In these patients, 1373 patients and 1372 patients had epithelial growth factor receptor (EGFR) mutation examination and anaplastic lymphoma kinase (ALK) mutation examination, respectively. We also analyzed whether the driver gene mutation status was similar between two groups and found that the proportion of patients having *EGFR* mutation was higher in FN groups (54.0% vs 45.6%, $P = 0.006$, **Figure 2**A). The similar trend was also observed in *ALK* aberration though the significance was at borderline ($P = 0.076$, Figure 2B). Then we compared the CTC level between gene (*EGFR* and *ALK*) mutant and wild type patients and found both *EGFR* mutant and *ALK* mutant patients have lower CTC level ($P_{EGFR} = 0.008$, $P_{ALK} = 0.002$, Figure 2C,D). Other clinicopathological characteristics were similar between FN and TP group (**Table 1**).

Univariate logistic analysis was also performed to explore factors correlating with the result of CTC test. In the analysis, we found patients being older (OR 1.209, 95% CI 1.029–1.420, $P = 0.021$), having larger tumor, more advanced T stage (OR 2.255, 95% CI 1.167–4.357, $P = 0.016$), lymph node metastasis (OR 1.520, 95% CI 1.085–2.131, $P = 0.015$), or advanced pathological stage (OR 1.794, 95% CI 1.181–2.274, $P = 0.006$) tended to be true positive in CTC detection. LUAD patients (OR 0.612, 95% CI 0.414–0.903, $P = 0.013$) and *EGFR* mutant patients (OR 0.712, 95% CI 0.559–0.906, $P = 0.006$) tended to be false negative in CTC detection (**Table 2**). And we selected the variables with *p*-value

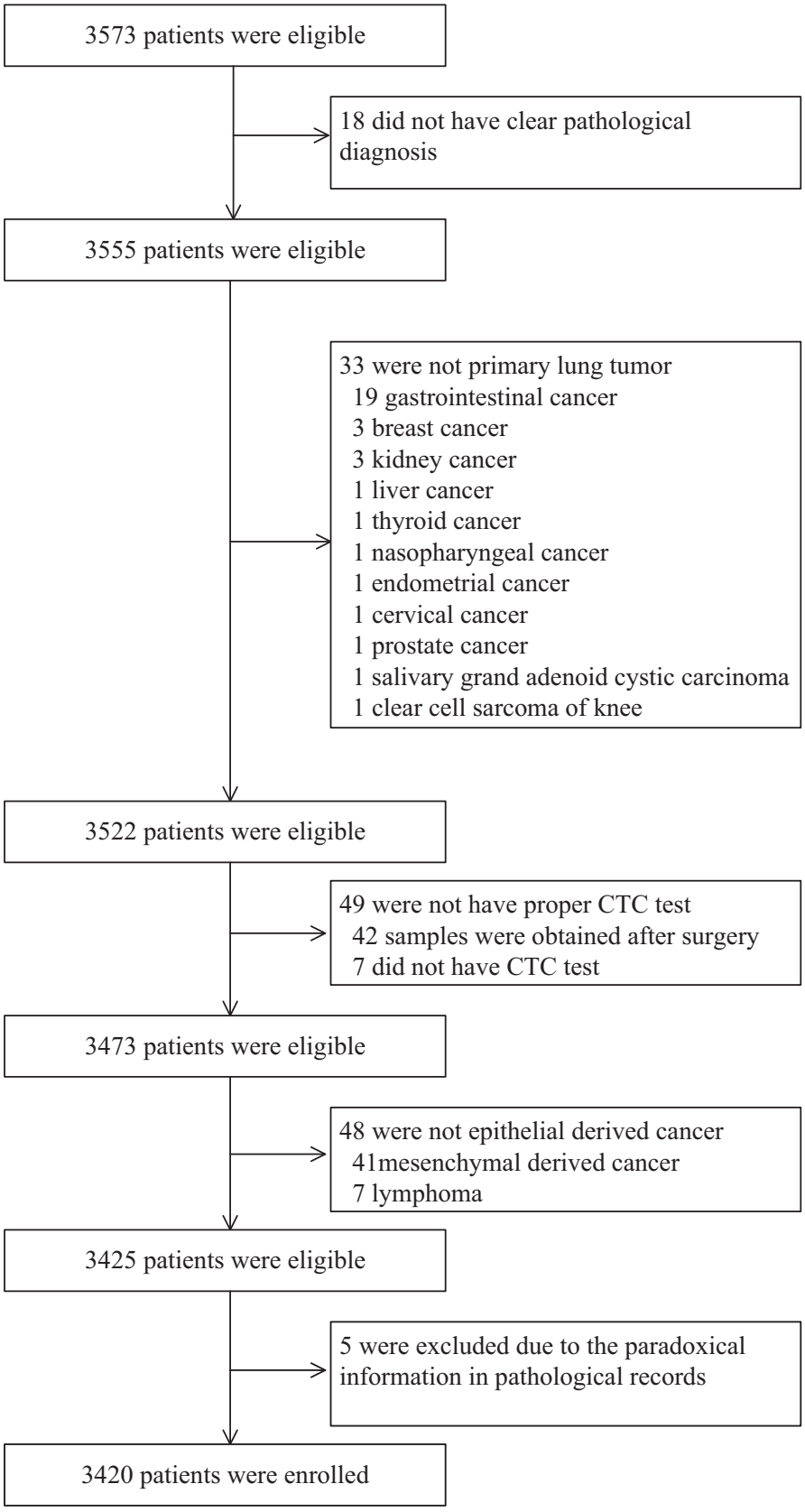

Figure 1. The flow chart of inclusion and exclusion of patients.

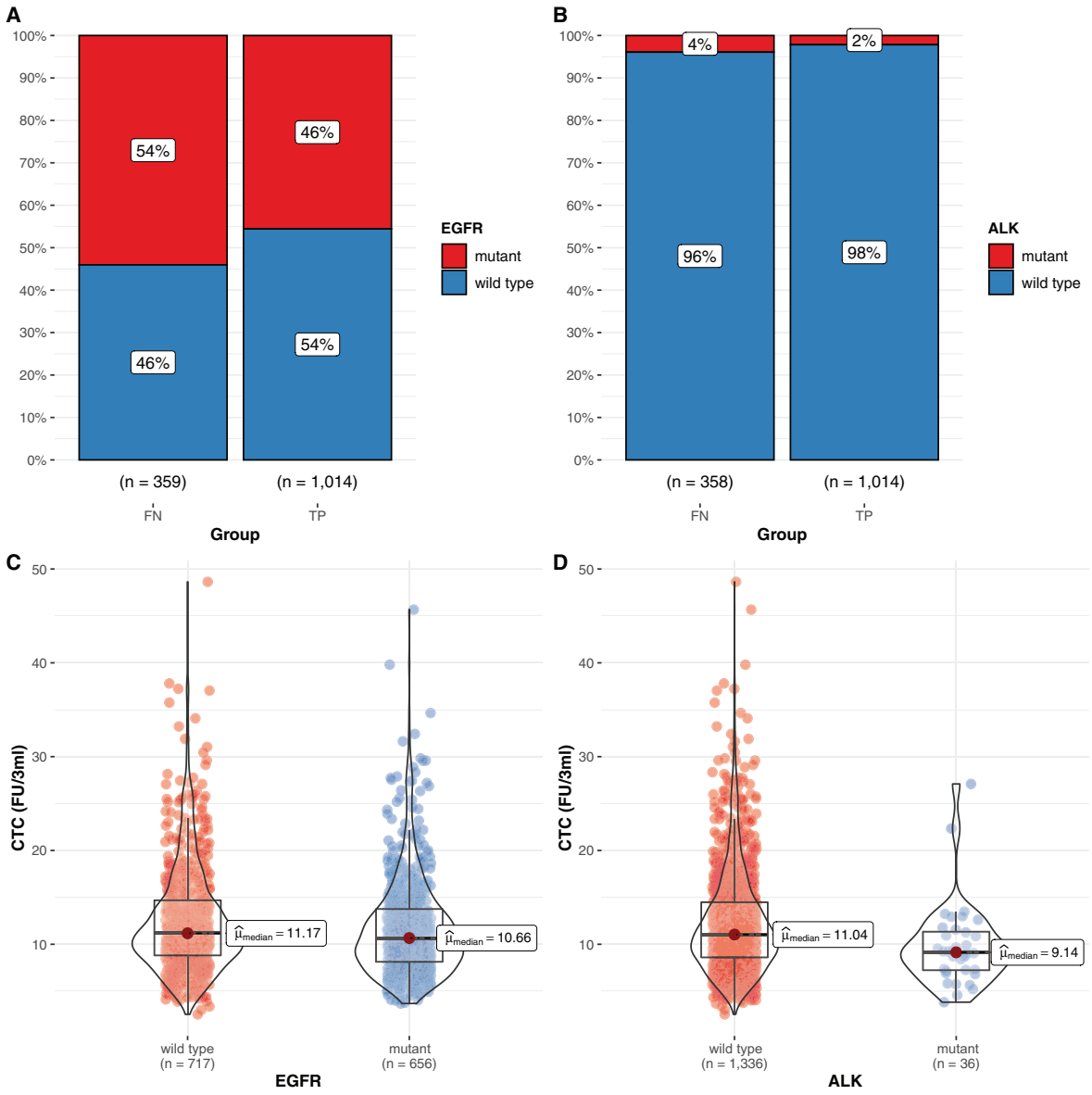

Figure 2. The correlation between *EGFR* /*ALK* mutation and CTC. A) *EGFR* mutation status in FN and TP group; B) *ALK* mutation status in FN and TP group; C) CTC level in *EGFR* mutant and wild type patients; D) CTC level in *ALK* mutant and wild type patients.

<0.05 for multivariate logistic analysis (As pathological stage was determined by T stage, N stage, and M stage, we excluded the pathological stage in multivariate analysis to avoid multicollinearity). After multivariate analysis, *EGFR* mutation status seemed to be an independent factor that had impact on CTC detection result, though the significance was at borderline (OR 0.795, 95% CI 0.613–1.031, $P = 0.083$). Other factors showed no significance in multivariate analysis (Table 2).

2.3. Subgroup Analysis

We extracted all LUAD and LUSC patients, two main pathological types of lung cancer, to analyze correlation of clinical features and CTC test result.

2.4. LUAD Patients with Different *EGFR* Mutation Subtypes Showed Difference in CTC Test

In our dataset, we totally had 2785 LUAD patients, in which 64.5% patients were female and 90.2% were non-smokers. Most patients were at the early stage, and only 4.0% patients were at advanced stage. When comparing FN and TP group, we found that the proportion of patients younger than 60 year old was significantly higher in FN group (59.7% vs 55.4%, $P = 0.041$, Supplementary Table S1). We also found that the composition of mutation subtype of *EGFR* was different between FN and TP group ($P = 0.005$, Table S1, Supporting Information) (The *EGFR* double mutation group contained three patients having L858R+T790M, one patient having 19del+T790M, one patient

Table 1. Clinicopathological characteristics of TP and FN group.

	True positive [TP] [$n = 2206$]	False negative [FN] [$n = 839$]	p-value
Gender			0.901
male	878 (39.8%)	336 (40.0%)	
female	1328 (60.2%)	503 (60.0%)	
Age			0.021*
<60	1180 (53.5%)	488 (58.2%)	
≥60	1026 (46.5%)	351 (41.8%)	
Smoking			0.353
never	1918 (86.9%)	740 (88.2%)	
ever	288 (13.1%)	99 (11.8%)	
Invasiveness#			0.446
pre-invasive	585 (26.5%)	234 (27.9%)	
invasive	1621 (73.5%)	605 (72.1%)	
Pathology			0.014*
LUSC	136 (6.2%)	33 (3.9%)	
LUAD	1994 (90.4%)	791 (94.3%)	
SCLC	16 (0.7%)	2 (0.2%)	
LCLC	23 (1.0%)	4 (0.5%)	
other	37 (1.7%)	9 (1.1%)	
STAS			0.626
negative	2047 (92.9%)	774 (92.4%)	
positive	157 (7.1%)	64 (7.6%)	
Tumor size			0.002*
≤3cm	1961 (88.9%)	781 (93.1%)	
3–5cm	187 (8.5%)	48 (5.7%)	
>5cm	58 (2.6%)	10 (1.2%)	
Pleural infiltration			0.112
negative	2119 (96.1%)	816 (97.3%)	
positive	87 (3.9%)	23 (2.7%)	
T stage			0.047*
Tis	585 (26.5%)	234 (27.9%)	
T1-2	1559 (70.7%)	594 (70.8%)	
T3-4	62 (2.8%)	11 (1.3%)	
Lymph node metastasis			0.014*
negative (N0)	2031 (92.1%)	794 (94.6%)	
positive (N1–3)	175 (7.9%)	45 (5.4%)	
M stage			0.830
M0	2195 (99.5%)	863 (99.6%)	
M1	11 (0.5%)	3 (0.4%)	
Pathological stage			0.019*
0	585 (26.5%)	234 (27.9%)	
I-II	1482 (67.2%)	574 (68.4%)	
III-IV	139 (6.3%)	31 (3.7%)	
EGFR			0.006*
wild type	552 (54.4%)	165 (46.0%)	
mutant	462 (45.6%)	194 (54.0%)	
KRAS			0.623
wild type	951 (94.5%)	338 (95.2%)	
mutant	55 (5.5%)	17 (4.8%)	

(*Continued*)

Table 1. (Continued).

	True positive [TP] [n = 2206]	False negative [FN] [n = 839]	p-value
BRAF			1.000
wild type	1003 (99.7%)	353 (99.7%)	
mutant	3 (0.3%)	1 (0.3%)	
ALK			0.076
wild type	992 (97.8%)	344 (96.1%)	
mutant	22 (2.2%)	14 (3.9%)	
ROS1			0.741
wild type	1010 (99.5%)	356 (99.2%)	
mutant	5 (0.5%)	3 (0.8%)	

LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; SCLC, small cell lung cancer; LCLC, large cell lung cancer; STAS, spread through air apace; EGFR, epidermal growth factor receptor; KRAS, KRAS proto-oncogene, GTPase; BRAF, B-Raf proto-oncogene, serine/threonine kinase; ALK, anaplastic lymphoma kinase; ROS1, ROS proto-oncogene 1, receptor tyrosine kinase; [#] We clarified carcinoma in situ as pre-invasiveness and other types as invasiveness; [*] $p < 0.05$

having L858R+20 insertion, four patients having G719X+S768I and one patient having G719X+L861Q.). After multiple comparison test, we found the proportion of L858R patients consisted lower proportion in TP group (P_{FDR} = 0.028). The proportion of 19del patients was much lower in FN group (P_{FDR} = 0.028) (**Figure 3**A).

Directly comparing of CTC level among different *EGFR* mutation subtypes showed that L858R patients had a relatively lower CTC level while 19del patients had a relatively higher CTC level though the statistical significance was not reached ($FDR_{19del\ vs\ WT}$ = 0.0656, $FDR_{L858R\ vs\ WT}$ = 0.071, Figure 3B). CTC level was also compared among different *KRAS* mutation subtypes, but no significant difference was found (Figure S1A, Supporting Information). Composition of mutation status of *KRAS* showed no difference between FN and TP group (Figure S1B, Supporting Information). As most patients did not have *KRAS* mutation, to avoid unbalance between *KRAS* WT and *KRAS* mutant groups, we further analyzed the composition of mutant subtypes of *KRAS* between FN and TP group, but no difference was found either (Figure S1C, Supporting Information).

We also compared that composition of dominant pathological subtype of invasive LUAD and no difference was found between FN and TP group (P = 0.905, Table S1, Supporting Information). According to the dominant pathological subtype, we divided invasive LUAD patients into low risk (lepidic), median risk (acinar and papillary), and high risk (micropapillary and solid). Neither composition of three risk levels between FN and TP group nor quantity of CTC among three risk levels was found different (**Figure 4**). But we observed a tendency that the CTC level gradually elevated from low risk to high risk (median CTC from 10.35 FU/3 mL to 11.12 FU/3 mL). Other clinical characteristics showed no difference between two groups (Table S1, Supporting Information).

In univariate logistic analysis, age (OR 1.190, 95% CI 1.007–1.407, P = 0.041) and *EGFR* mutation status (OR 0.645, 95% CI 0.477–0.873, P = 0.005) showed significance, and they were included in multivariate logistic analysis. The result showed that LUAD patients with *EGFR* L858R mutation would be more likely to be negative in CTC examination (OR 0.625, 95% CI 0.461–0.848, P = 0.003, Table S2, Supporting Information). Patients with *EGFR* uncommon mutation had a similar tendency, though the significance was at borderline (Table S2, Supporting Information).

2.5. Clinicopathological Characteristics of LUSC

In our dataset, we extracted 169 LUSC patients totally. In these patients, 95.9% patients were male, 49.7% were smokers and 20.7% patients were at advanced stage. After comparing the clinical features between TP and FN group, we failed to find any significant differences (Table S3, Supporting Information). Univariate logistic analysis also did not find any significant factors that could influence that result of CTC test (Table S4, Supporting Information).

3. Discussion

In this study, we comprehensively compare the clinicopathological characteristics between FN and TP group in FR-base CTC test in lung carcinoma. In the whole dataset, we found that age, tumor size, pathological type, T stage, N stage, pathological stage, and *EGFR* mutation status were different between FN and TP group. In LUAD subgroup, age and *EGFR* mutation subtype were found to be different between two groups, while in LUSC subgroup no feature was found significantly different between two groups. Moreover, we also reported that the sensitivity of FR-based CTC detection in diagnosing lung carcinoma was 72.4%.

CTCs were shed from a solid tumor, which could be from primary site or metastatic site, and circulated in vasculature as single cells or aggregated as clusters, which were reported in many cancers.[7,9] With the development of detection and enrichment technique, CTC has become an important resource of liquid biopsy.[16] In diagnosis of lung cancer, the sensitivity of CTC showed a wide range, which might be caused by different detection strategies used in studies. A study used physical property based CTC detection method, ISET, only showed 26.3% sensitivity in lung cancer detection,[10] which was significantly lower than the method we used. The sensitivity of biological property base CTC detection methods in lung cancer diagnosis was ranged

Table 2. Logistic regression analysis of clinicopathological characteristics and FR-based CTC test result.

	univariate logistic regression			multivariate logistic regression		
	OR	95%CI	*p*-value	OR	95%CI	*p*-value
Age						
<60	–	–	Reference	–	–	Reference
≥60	1.209	1.029–1.420	0.021*	1.221	0.954–1.563	0.113
Gender						
female	–	–	Reference			
male	0.990	0.841–1.164	0.901			
Smoking						
never	–	–	Reference			
ever	1.122	0.880–1.432	0.353			
Invasiveness#						
pre-invasive	–	–	Reference			
invasive	1.072	0.897–1.281	0.446			
Pathology						
LUSC	–	–	Reference	–	–	Reference
LUAD	0.612	0.414–0.903	0.013*	0.864	0.534–1.396	0.549
SCLC	1.941	0.425–8.861	0.392	4.214	0.527–33.693	0.175
LCLC	1.395	0.452–4.310	0.563	1.426	0.450–4.525	0.547
other	0.998	0.439–2.269	0.995	1.882	0.643–5.126	0.259
STAS						
negative	–	–	Reference			
positive	0.928	0.686–1.255	0.626			
Tumor size						
≤3cm	–	–	Reference	–	–	Reference
3–5cm	1.552	1.118–2.153	0.009*	1.232	0.847–1.793	0.275
>5cm	2.310	1.175–4.542	0.015*	2.344	0.199–27.555	0.498
Pleural infiltration						
negative	–	–	Reference			
positive	1.457	0.914–2.323	0.114			
T stage						
Tis	–	–	Reference	–	–	Reference
T1-2	1.050	0.878–1.255	0.594	0.000	–	1.000
T3-4	2.255	1.167–4.357	0.016*	0.000	–	1.000
Lymph node metastasis						
negative (N0)	–	–	Reference	–	–	Reference
positive (N1–3)	1.520	1.085–2.131	0.015*	1.151	0.787–1.684	0.467
M stage						
M0	–	–	Reference			
M1	1.397	0.389–5.018	0.609			
Pathological stage						
0	–	–	Reference			
I-II	1.033	0.863–1.236	0.725			
III-IV	1.794	1.181–2.274	0.006*			
EGFR						
wild type	–	–	Reference	–	–	Reference
mutant	0.712	0.559–0.906	0.006*	0.795	0.613–1.031	0.083

(*Continued*)

Table 2. (Continued).

	univariate logistic regression		multivariate logistic regression
KRAS			
wild type	–	–	Reference
mutant	1.150	0.658–2.009	0.624
BRAF			
wild type	–	–	Reference
mutant	1.056	0.109–10.183	0.963
ALK			
wild type	–	–	Reference
mutant	0.545	0.276–1.077	0.081
ROS1			
wild type	–	–	Reference
mutant	0.587	0.140–2.471	0.468

LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; SCLC, small cell lung cancer; LCLC, large cell lung cancer; STAS, spread through air apace; EGFR, epidermal growth factor receptor; KRAS, KRAS proto-oncogene, GTPase; BRAF, B-Raf proto-oncogene, serine/threonine kinase; ALK, anaplastic lymphoma kinase; ROS1, ROS proto-oncogene 1, receptor tyrosine kinase; # We clarified carcinoma in situ as pre-invasiveness and other types as invasiveness; $^{*}p < 0.05$

from 68.29% to 85.7%.[13,15a-c,17] Studies also suggested that combined with traditional blood tumor biomarkers could further improve the sensitivity of CTC in lung cancer diagnosis.[15a] As for FR-based CTC examination, Li et al. reported the sensitivity of the method was 85.7% that was ≈10% higher than that in our study.[15a] This discrepancy might be caused by different cancer subtype and cutoff value. Another two studies in lung cancer reported the overall sensitivity as 74.4% and 77.7%,[15b,c] respectively, which was slightly higher than the results of our study. As study had demonstrated that sensitivity could be varied from stage I to stage IV[15b] and only 5.6% patients in our study had advanced lung cancer, the higher sensitivity might be due to relatively higher proportion of patients with advanced stage cancer in their studies.

In our study, we found patients at stage 0–II were tended to be FN in FR-based CTC test comparing to those at stage III-IV. The result was in accordance with another study as the diagnostic yield of the test was 69.8% that was significantly lower than that in stage IV (90.6%).[15b] Meanwhile, we also found that patients with tumor no larger than 3 cm and low T stage would be more likely to be FN in the test. The similar trend was reported in another study though the difference was not significant.[15a] Moreover, Chen et al. have demonstrated that CTC level could be significantly lower in stage I-II.[15c] It indicated that solely using CTC to distinguish lung carcinoma from benign disease in patients having abnormality on CT image, especially having small lesion, should be cautious. Due to lymphatic drainage, carcinoma cells in lymph node might disseminate into blood more easily. Thus, lymph node metastasis was also associated with the positive result of CTC test in our study. However, we did not find any correlation between M stage and CTC test that might be caused by a few patients were M1 in our cohort. Pathological composition was also different between FN and TP group, and LUSC consisted less in FN group. Another study reported 72.34% LUSC patients were positive in CTC test while only 68.14% LUAD patients were positive in CTC test.[15a] However, there were studies suggested that CTC level was not correlated with pathological type of lung carcinoma.[15b,c] Thus, the relationship between pathological type and CTC in lung carcinoma still need further study. Furthermore, we reported an association between *EGFR* mutation status and the result of CTC test. In all patients, a lower CTC level was found in *EGFR* mutant group. In LUAD subgroup, after further dividing *EGFR* mutation status, we suggested that patients having *EGFR* 19del had relatively higher CTC level while *EGFR* L858R patients had relatively lower CTC level than *EGFR* wild type patients, respectively. But the reason was still unclear. As studies have used CTC to analyze the mutation status of cancer or guide the target therapy,[18] it should be cautious whether some tumor with specific mutation would be difficult to obtain enough CTCs and test result of some specific mutations would be false negative by analyzing CTCs.

CTC detection could also be applied in predicting cancer prognosis or treatment efficacy. Kanayama et al. reported that baseline CTC-positive lung cancer patients had a worse survival no matter being treatment with surgery or chemotherapy.[11] Another study also reported a shorter disease free survival (DFS) in patients with higher CTC level.[13] A study analyzed CTC level at multiple timepoint during chemotherapy indicated that no matter at that time point the positive result foreshadowed a worse outcome of non-small cell lung cancer (NSCLC) patients. Progression free survival (PFS) and overall survival (OS) would significantly shorten in patients with persistent positive result of CTC test.[19] There are studies constructed classifiers based on copy number variation of CTCs to identify chemosensitive and chemorefractory small cell lung cancer (SCLC).[20] For immune checkpoint inhibitor, pre-treatment or 3 month after treatment, occurrence of programmed cell death ligand 1 (PD-L1)(+) CTC indicated a worse prognosis of nivolumab in NSCLC.[21] Higher PD-L1(+) CTC was found in nivolumab non-respond group and PD-L1(+) CTC would occur in all NSCLC patients after progression.[22] A study suggested that most mutations of primary and metastatic sites of SCLC could be detected by single-cell sequencing of CTC that indicated this approach could provide a mutational profile of SCLC in a noninvasive way.[20b] This might be used to evaluate

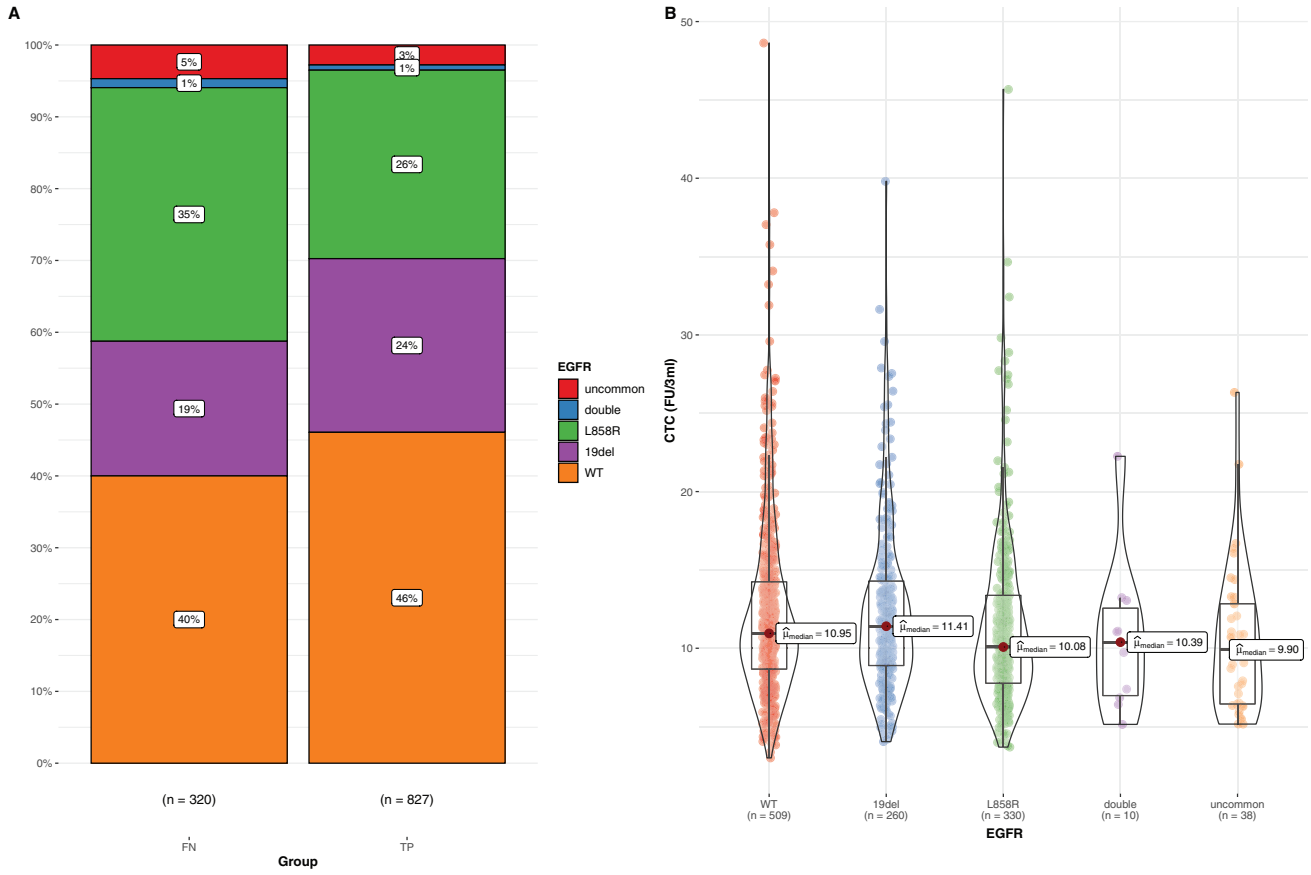

Figure 3. CTC level and *EGFR* mutation status in LUAD patients. A) composition of different *EGFR* mutation in FN and TP group; B) CTC level of LUAD patients having different *EGFR* mutation.

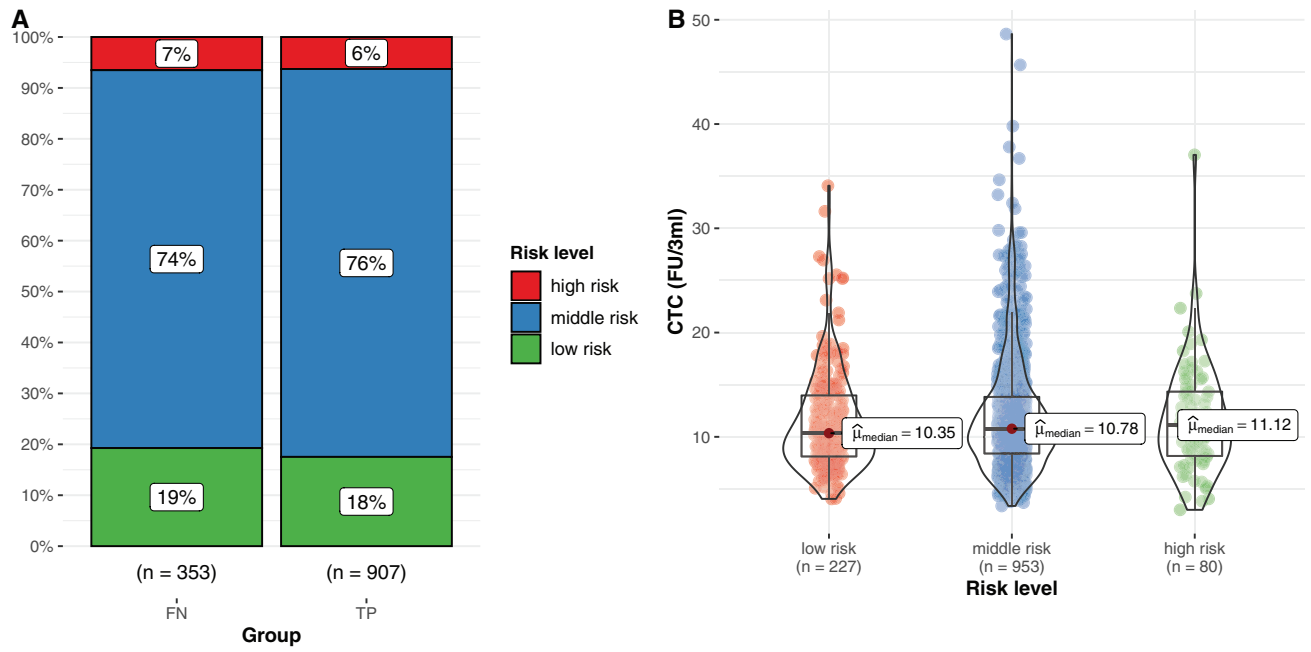

Figure 4. CTC level among invasive LUAD patients having different dominant pathological subtype. A) the composition of patients at different risk levels in FN and TP group; B) CTC level among different risk levels. (low risk: lepidic, median risk: acinar and papillary, high risk: micropapillary and solid).

tumor mutation burden (TMB), and might be a biomarker for programmed cell death 1 (PD-1)/PD-L1 inhibitor treatment in SCLC.[20b] Furthermore, Lin et al. suggested that CTC could be a biomarker for predicting the efficacy of nature killer (NK) cell treatment in stage IV NSCLC.[23] As patients in our dataset were all had surgery and survival data were not applicable at current, we could not explore the correlation between CTC and surgery outcome in the study.

There are some limitations in our study. First, this is a single-center retrospective study that means it is inevitable to avoid all unmeasured confounders. Second, the dataset contains limited variables, which need to be supplemented and validated by prospective studies. Third, survival data are not applicable at present that limits us to analyze the survival of patients with different CTC level, and we may analyze it in the future.

4. Conclusion

In our current study, we comprehensively analyzed the clinicopathological features of lung carcinoma patients having true positive results and false negative results in FR-based CTC test. We found patients with smaller tumor, earlier T stage, earlier pathological stage, without lymph node metastasis, LUAD subtype and *EGFR* mutation would be more likely to have false negative result in FR-based CTC test. Further multi-center prospective studies were needed for further confirmation of these findings.

5. Experimental Section

Study Design: This was a single institution retrospective study. Totally, 3573 patients were suspected of lung cancer due to the lumps, spots, or shadows on their CT image and they underwent surgery in Shanghai Pulmonary Hospital from June 2018 to March 2019. The inclusion criteria are listed below: 1) having a clear pathological diagnosis; 2) primary lung tumor; 3) having CTC examination and the blood sample was obtained before surgery; 4) epithelial derived lung cancer; 5) the pathological record was not paradoxical. The exclusion criteria were as following: 1) do not have a clear pathological diagnosis or the information in the record was paradoxical or confused; 2) the lesion of lung was relapse or metastasis of other cancer; 3) mesenchymal derived lung cancer or lymphoma; 4) do not have CTC examination before surgery. Pathological classification was according to the 2015 World Health Organization Classification of lung cancer and staging was according to the International Association for the Study of Lung Cancer (IASLC) Eighth Edition of tumor node metastasis (TNM) classification of lung cancer.[24]

The study was conducted in accordance with the Declaration of Helsinki. And this study was approved by Ethic Committee of Shanghai Pulmonary Hospital (No.K21-113Y).

Date Collection: Age, gender, smoking, and the clinicopathological information (invasiveness, tumor size, pleural infiltration, lymph node metastasis, pathological stage, TNM classification), and gene mutation status (including *EGFR* mutation, *KRAS* mutation, *BRAF* mutation, *EML4-ALK* fusion, and *ROS1* fusion) were extracted from the medical records.

FR$^+$CTC Detection: *Sample Preparation and CTC Enrichment*: Peripheral blood (3 mL) would be collected in ethylene diamine tetraacetic acid (EDTA) anticoagulation tube from patients. The samples would be processed within 24 h or be stored at 4 °C. CTCs were enriched by CytoploRare Folate Receptor Positive Circulating Tumor Cell Detection Kit (GenoSaber Biotech Co. Ltd., Shanghai, China) and all procedures were according to the manufactural protocol as previous studies[15c,25] Briefly, 12 mL lysis buffer would be added into blood samples at first to deplete erythrocytes. Then cluster differentiation antigen 45 (CD45) and cluster differentiation antigen 14 (CD14) binding immunomagnetic beads would be added to enrich and eradicate leukocytes and CTCs could be preserved in the left cells.

FR Labeled and Quantified: Add 100 µL activating buffer and incubate on ice for 1 min to activate the cells. Then the sample would be centrifuged at 600 g, 4 °C for 10 min. Later, 10 µL folic acid conjugated oligonucleotide probe (5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGTTCTAA-3′) would be incubated with activated cells at room temperature for 40 min. After terminating the reaction with 900 µL washing buffer, cells would be centrifuged at 600 g, 4 °C for 10 min. Wash the cells with washing buffer for three times and collect the cells. After that, use 120 µL elution buffer to resuspend the cells and incubate 2 min on ice. Ultimately, centrifuge the incubated samples (600 g, 4 °C, 10 min) to obtain the supernatant and add 20 µL neutralizing buffer for later quantification.

Quantification was performed through a Taqman probe-based quantification PCR (qPCR). The primers used are listed as follows: forward primer, 5′-TATGATTATGAGGCATGA-3′; reverser primer, 5′-GGTGTCGTGGAGTCG-3′; Taqman probe, 5′-FAM-CAGTTGAGGGTTC-MGB-3′. QPCR was run on 7300 Plus Real-Time PCR system (Applied Biosystem): 2 min at 95 °C, 30 s at 40 °C, 1 min at 60 °C, 5 min at 8 °C, 1 min at 95 °C, 40 cycles of 10 s at 95 °C, 30 sec at 35 °C,5 s at 72 °C, and signals were captured during annealing step (35 °C). According to the manufacture, result <8.70 FU 3 mL^{-1} would be defined as CTC negative.

The patients confirmed as epithelial lung cancer by pathological examination and CTC positive by FR-based CTC detection were defined as TP group. While the patients who had epithelial lung cancer, however, CTC negative by FR-based CTC detection were defined as FN group.

Statistical Analysis: Continuous variables were show, as median (interquartile range), and categorical variables were shown as number (proportion). Mann–Whitney *U*-test or Kruskal–Wallis Rank Sum test was used for comparing continuous data between two groups or among multiple groups. Chi-square test or Fisher exact test was used for comparing categorical data. False discovery rate (FDR) was applied for adjustment of multiple comparison. *p*-value or FDR <0.05 would be considered as significant. Data analysis was performed through SPSS 22.0 (SPSS Inc., Chicago, USA) and R software (version 4.1.0) in R studio (Boston, USA). Figures were generated by ggplot2 or ggstatsplot package.[26]

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

Keywords

clinicopathological characteristics, false negative, folate receptor based circulating tumor cell detection, lung cancer, true positive

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