Original Article

Correlation of pulmonary venous circulating tumor cells with clinicopathological parameters in patients with early-stage lung adenocarcinoma

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Background: Tumor recurrence following the surgical resection of lung cancer (LCa) reduces long-term disease-free survival rates. This study aimed to investigate the association of pulmonary venous blood (PVB) circulating tumor cells (CTCs) with the clinicopathological features of patients with early-stage lung adenocarcinoma (ESLA).

Methods: A total of 120 cases were enrolled, including 24 healthy controls, 36 patients with lung benign tumors, and 60 ESLA patients. Cells displaying a profile of human chromosome 8 specific sequence $(CEP8)^+$/4',6-diamidino-2-phenylindole $(DAPI)^+$/leukocyte-specific antibodies $(CD45)^-$ were regarded as CTCs, and counts of ≥2 CTCs per 3.2 mL of PVB were considered positive. The association of CTC counts with clinical parameters were analyzed.

Results: The number of CTCs were significantly higher in ESLA patients compared to benign or normal control group. Moreover, increased CTCs in lung adenocarcinoma was closely associated with tumor invasion, pathological staging and the epidermal growth factor receptor (*EGFR*) mutations ($P<0.05$), whereas no significant difference was observed between CTC counts and age, sex, smoking history, pathological cell morphology or immunohistochemical indicators ($P>0.05$). Univariate and multivariate Cox's proportional hazards regression confirmed that CTC counts were an independent indicator for the prediction of tumor invasion, pathological staging, and *EGFR* mutations.

Conclusions: Our data suggest that CTC counts correlate with tumor invasion, pathological staging, and *EGFR* mutations. CTCs therefore represent promising biomarkers for the surveillance of lung adenocarcinoma progression.

Keywords: Circulating tumor cells (CTCs); early-stage lung adenocarcinoma (ESLA); clinicopathological factors; epidermal growth factor receptor (*EGFR*)

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Introduction

Lung cancer (LCa) is one of the most common global malignancies and a leading cause of cancer related death (1). Most LCa patients are diagnosed when tumor metastasis has already occurred, and despite curative treatment, postoperative metastases and recurrence are frequent. When diagnosed during the early stages (stage I), ~25–50% of patients experience recurrence or distant metastasis within

5 years (2,3). The progression of early-stage LCa is distinct and varied from micro to occult metastases and recurrence. Although serologic tumor markers, clinicopathological parameters, and radiologic modalities are commonly used in the clinical management of LCa patients, disease progression, surveillance, and therapeutic responses are difficult to monitor (4-6). In this regard, the identification of novel biomarkers for non-invasive LCa detection would permit the integration of tumor prediction and real-time surveillance, improving therapeutic and clinical outcomes.

Accumulating evidence indicates that circulating tumor cells (CTCs) from primary tumors serve as a primary driving force for tumor progression, metastasis, and chemotherapy resistance (7-12). CTCs can be detected in most solid tumors and correlate with prognosis, tumor metastasis, and relapse (13-19). To-date, CTC detection has been employed in colon cancer, breast cancer and prostate cancer studies (13-17). Despite their promising clinical relevance, only 23.7% of non-small cell lung cancer (NSCLC) patients exhibit positive CTC detection (>1 per 7.5 mL of blood) in Chinese and Western populations (20). CTCs were also undetectable in the peripheral blood (PB) of stage I LCa patients.

The analysis of CTC counts in PB *vs.* pulmonary venous blood (PVB) demonstrated higher numbers of CTCs from PVB samples, implying a higher sensitivity of CTC detection for early stage LCa (6,21,22). In this study, we prepared CTCs from PB and PVB in cohort of 120 subjects containing 24 healthy controls (collecting PB), 36 patients with benign lung tumors (collecting intraoperative PVB) and 60 early-stage lung adenocarcinoma (ESLA) patients (collecting intraoperative PVB), to evaluate the association between CTC counts and clinicopathological features. Herein, we highlight PVB CTCs as biomarkers for the real-time surveillance of LCa progression and therapy.

Methods

Patients and sample collection

A total of 60 ESLA patients diagnosed in our hospital from January 2017 to December 2017 were enrolled. The diagnosis of ESLA was based on the Guidelines on the Clinical Management of LCa (2017 edition). There were 32 males and 28 females, aged 37–77 years (median =55 years). Pathological stages were determined according to current tumor-node-metastasis (TNM) classification as revised in 2018 (23). Pathological staging included 10 cases in stage IA1, 18 cases in stage IA2, and 32 cases in stage IA3. The degree of infiltration was classified into adenocarcinoma *in situ*, micro-invasive adenocarcinoma, and invasive adenocarcinoma. From biopsy specimens, pathological cell types were classified according to the 2015 WHO classification criteria. Pathological cell morphology was divided into wall-like, acinar, micro-papillary, and papillary. The association between immunohistochemical staining and genetic mutations in Napsin A, TTF-1, Ki67 and epidermal growth factor receptor (*EGFR*) were also assessed.

The patients underwent lung resection via open thoracotomy or video-assisted thoracoscopic surgery, with wedge resection followed by lobectomy. PVB was drained from the affected lobe which was isolated using minimal manipulation of the lung. Up to 10 mL of PVB was removed using a 22-gauge needle attached to a 10 mL syringe (22-gauge spinal needle was employed for video-assisted thoracoscopic surgery) prior to removal of the lesion (intraoperative PV). Heparin was added to all blood samples to prevent coagulation and cancer cells were detected within 2 h of collection. PB samples were collected from the control group.

Enrichment and identification of ESLA patients CTCs

The enrichment of ESLA CTCs was performed as previously described (24,25) in a blood volume of 3.2 mL. Briefly, red blood cells (RBCs) in the PVB samples were lysed, and residual cell pellets were resuspended in PBS. Samples were labeled with anti-leukocyte-specific antibodies (CD45) monoclonal antibody coated magnetic beads for 30 min, followed by separation of the beads using a magnetic stand. LCa CTCs were identified by CD45-FISH which combined FISH labeling with chromosome 8 centromere probes and anti-CD45 monoclonal antibodies. Briefly, specimens were hybridized with human chromosome 8 specific sequence (CEP8) probes at 37 ℃ for 20 min and washed in 50% formamide at 43 ℃ for 20 min. Samples were immersed into 2 × SSC gradient alcohol, and washed twice with 0.2% bovine serum albumin (BSA). Specimens were labeled with anti-CD45 antibodies conjugated to Alexa Fluor 594 in 2% BSA for 1.5 h. Specimens were washed in 0.2% BSA and mounted in Vectashield containing 4',6-diamidino-2-phenylindole (DAPI). Fixed samples were imaged along the "S" track. For the classification of positive CTCs, cells must be hyperdiploid and $CEP8^+/DAPI^+/CD45^-$.

CTC quantification and positive cut off values

CTCs ≥2 suggested the presence of malignant cells, suspected malignant lesions, and a potential risk of metastasis or distant metastasis (25). CTCs <2 suggested normal levels and that the nature of the lesion was benign. We referred to existing clinical diagnosis and treatment standards for all comprehensive assessments (26).

Primary tissue collection, genotyping of EGFR gene mutations (ARMS)-polymerase chain reaction (PCR) assays

DNA was extracted from formalin-fixed, paraffin-embedded lung tumor tissues using QIAamp DNA FFPE Tissue Kits. *EGFR* mutations were detected using the AmoyDx Human *EGFR* Gene 29 mutation detection kit with fluorescence PCR. Assays were performed on an ABI7500 real-time PCR instrument. Primers were labeled with 6-carboxyfluorescein and HEX. The *EGFR* kit detects 29 mutations in exons 18 to 21, including T790M, L858R, L861Q, S768I, G719S, G719A, and G719C; three insertions in exon 20; and 19 deletions in exon 19. DNA was PCR amplified in a final volume of 25 μL. PCR reactions contained 5 μL of DNA, 25 mmol/L $MgCl_2$, 25 mmol/L dNTP, 100 μmol/L of forward and reverse primers, 10 X Takara buffer, and 5 U/μL Takara HS-Taq. The first cycle of amplifications was performed with a 5 min initial denaturation at 95, followed by 30 cycles of 45 s at 95 ℃, 45 s at 54 ℃, 1 min at 72 ℃, and a 6 min final extension at 72 ℃. Products from the first cycle were amplified in secondary cycles using identical PCR conditions.

Statistical analysis

Individual variables were assessed by univariate analysis using the Chi squared test. Risk ratios were calculated for each variable to assess the predictive values for CTCs. Logistic regression analysis was used to assess the relationships between CTC counts and clinicopathological data. All statistical analyses were performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Graphs were plotted using GraphPad Prism (Version 5.02. San Diego, CA, USA). The area under each ROC curve (AUC) was calculated to assess the discriminating power. The Youden index (sensitivity + specificity) was calculated to select the optimal cut-off values for CTC distribution. P<0.05 was considered a statistically significant difference.

Results

Identification and characterization of CTCs enriched from PVB

CTCs were isolated and characterized as previously reported. Briefly, CTCs enriched from PVB were subjected to immunofluorescence in situ hybridization (imFISH) staining with antibodies against CEP8 and CD45. Cell nuclei were stained with DAPI. CTCs were defined as cells showing a $CEP8^+/DAPI^+/CD45^-$ profile. CTC counts less than 2 were considered false positives (*Figure 1A*,*B*,*C*,*D*,*E*,*F*). Viability assessments suggested that the CTCs isolated from PVB were approximately similar to those of whole blood cells (*Figure 1G*). Following this criterion, no positive CTCs were detected in either healthy controls or benign lung disease patients. In contrast, CTCs were identified in up to 73.33% (44/60) of patients with ESLA (P<0.05; *Table 1*), indicating these cells as specific signatures for early stage lung adenocarcinoma (*Figures 2*,*3*).

CTC counts positively correlate with tumor invasion and pathological stage

Through the evaluation of CTC counts with clinicopathological features, CTCs were found to be associated with tumor infiltration (P=0.003) and pathological stage (P<0.000), (*Table 2*). The AUC analysis indicated that CTC counts >5 could predict tumor infiltration and higher pathological staging (specificity 85%, sensitivity 90%), whilst CTC counts <3 were associated with *in situ* IA1 staging. Of note, no correlation between CTCs and other factors such as gender, age, smoking history, pathological cell morphology, and immunohistochemical indicators were observed. These findings demonstrate that CTC counts are associated with tumor infiltration and pathological stage in patients with early stage lung adenocarcinoma.

Relationship between CTCs and EGFR gene mutations

EGFR mutations are commonly observed in ESLA patients (27). Inhibitors targeting the kinase domain of *EGFR*, particularly tyrosine kinase inhibitors (TKIs), are in clinical use and show therapeutic efficacy (28,29). We therefore examined the relationship between CTC counts and *EGFR* mutations. DNA was extracted from formalin-fixed, paraffin-embedded lung tumor tissues and *EGFR* mutations were detected using the AmoyDx Human *EGFR*

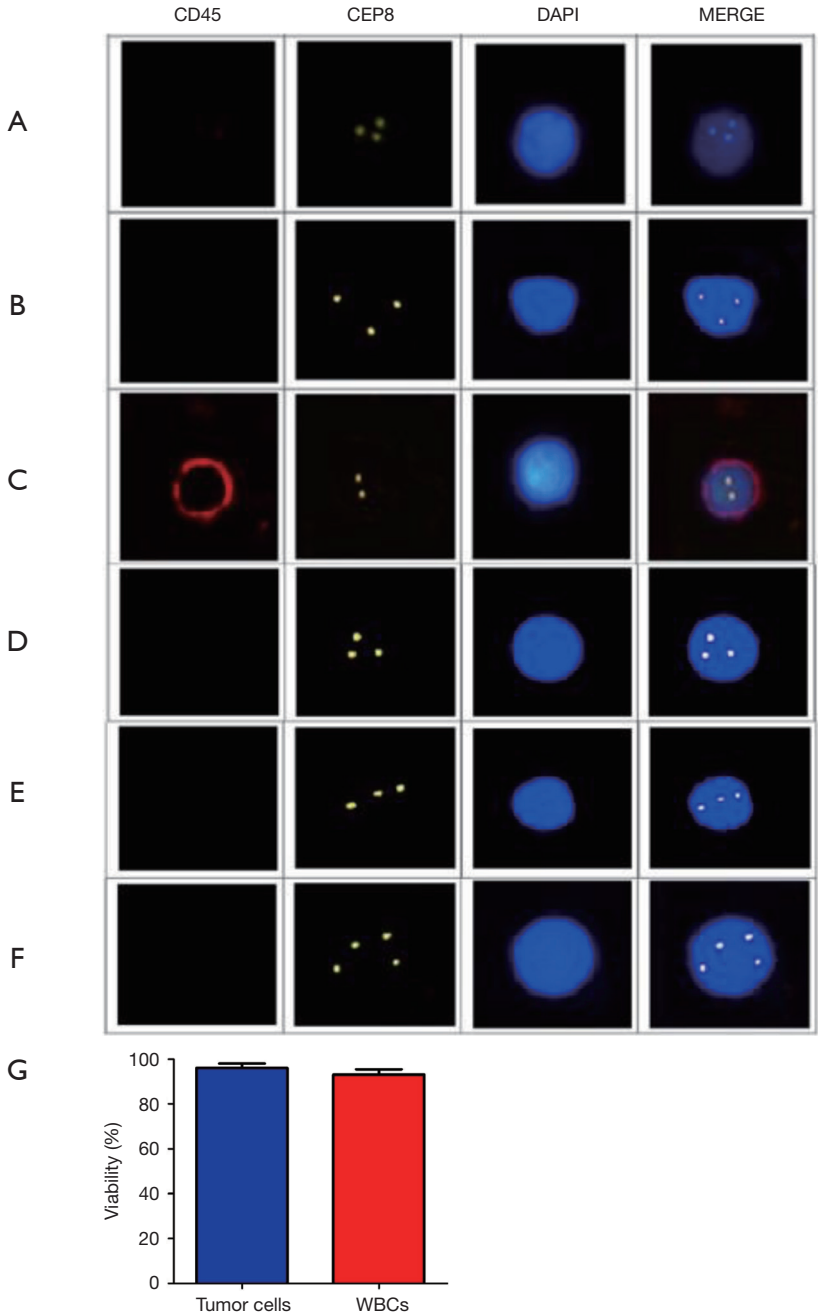

Figure 1 Immunostaining of early-stage lung adenocarcinoma patients CTCs enriched from patient pulmonary venous blood, 400×. CTCs enriched from early-stage lung adenocarcinoma patients were subjected to imFISH staining. Only CTCs was specifically immunostained with anti-human CEP8, but not for anti-human CD45 staining. (A) Positive control group; (B) CTC =7; (C) CTC =0; (D) CTC =3; (E) CTC =2; (F) CTC =1; (G) average of viability of the enriched tumor cells (blue) and WBCs (red) is 96.03%±3.42% and 93.03%±4.14% (mean ± SD), respectively. Results represent average of values obtained in 3 separate experiments performed in triplicate. Viability examination of tumor cells and WBCs following subtraction enrichment and immunostaining processing. Necrotic lung adenocarcinoma cell ($CD45^+$/$CEP8^+$/$DAPI^+$), and WBC ($CD45^+$/$CEP8^-$/$DAPI^+$) are depicted. Healthy cells are CD45 negative. Due to the above picture space is limited, did not show all the results. CTCs, circulating tumor cells; imFISH, immunofluorescence in situ hybridization; CD45, leukocyte-specific antibodies; CEP8, human chromosome 8 specific sequence; DAPI, 4',6-diamidino-2-phenylindole; MERGE, CD45 + CEP8 + DAPI; WBCs, white blood cells.

Table 1 Early-lung adenocarcinoma and benign lung disease and healthy controls differences in circulating tumor cells

Variable	n	Positive/negative	Positive-CTC (%)	χ^2	P value
Early-lung adenocarcinoma group	60	44/16	73.33	69.474	<0.05
Benign lung disease group	36	0/36	0		
Healthy controls group	24	0/24	0		

CTC, circulating tumor cell.

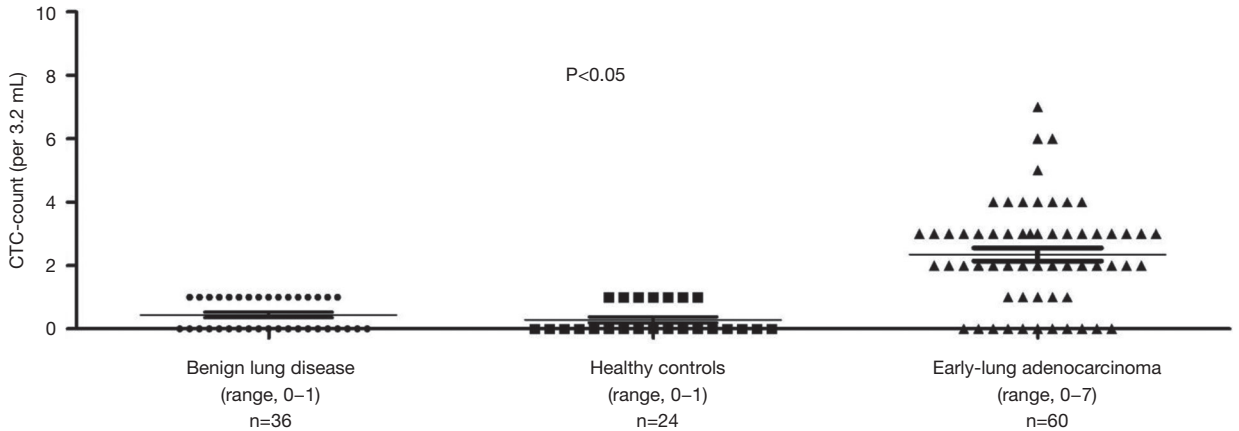

Figure 2 Distribution of CTCs count in early-lung adenocarcinoma group patients (n=60, range, 0–7), benign lung disease patients (n=36, range, 0–1) and healthy controls (n=24, range, 0–1). Graphs were plotted by means of the GraphPad Prism (Version 5.02. San Diego, CA, USA), P<0.05. CTCs, circulating tumor cells.

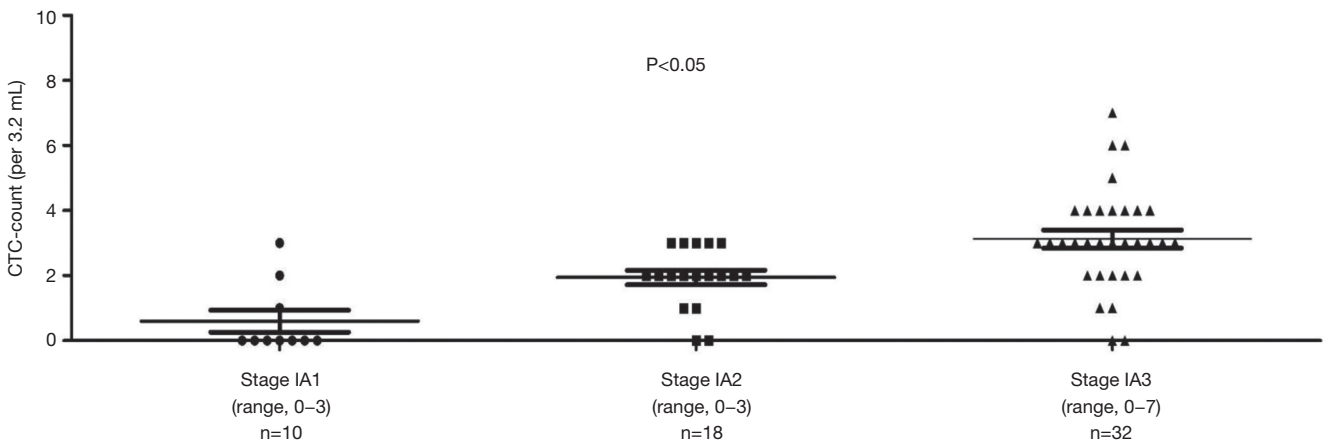

Figure 3 Distribution of CTCs count in patients with early-lung adenocarcinoma patients according to pathological stage. Graphs were plotted by means of the GraphPad Prism (Version 5.02. San Diego, CA, USA), P<0.05. CTCs, circulating tumor cells.

Gene 29 mutations detection kit (*Figure 4*). The results showed positive CTC rates of 53.33% in patients with *EGFR* negative lung adenocarcinoma, and 93.55% positive CTC rates in patients with *EGFR* mutations (*Table 3*; P<0.05). CTCs were present in 26.67% of patients with L858R point mutations, and 28.33% of patients with exon 19 deletions (the two most common *EGFR* genetic alterations) (*Table 4*). No *EGFR* mutations were detected in

Table 2 Circulating tumor cells and gender, age, smoking history, degree of tumor invasion, pathological cell morphology, pathological staging and immunohistochemical indicators

Clinicopathological parameters	n	Positive/negative	Positive-CTC (%)	χ^2	P value
Gender				0.097	0.491
Male	32	24/8	75.00		
Female	28	20/8	71.43		
Age (years)				0.128	0.473
>55	36	27/9	75.00		
≤55	24	17/7	70.83		
Smoking history				0.302	0.402
Never smoked	26	20/6	76.92		
Have or are smoking	34	24/10	70.59		
Degree of tumor infiltration				11.851	0.003
In situ cancer	6	1/5	16.67		
Micro-infiltration	22	16/6	72.73		
Infiltration	32	27/5	84.38		
Pathological cell morphology				5.313	0.150
Wall-like	8	4/4	50.00		
Acinar	22	19/3	86.36		
Micro-papillary	12	10/2	83.33		
Papillary	18	11/7	61.11		
Pathological staging				18.011	0.000
IA1	10	2/8	20.00		
IA2	18	14/4	77.78		
IA3	32	28/4	87.50		
Immunohistochemical indicators				3.561	0.313
NapsinA(+)/TTF-1(+)/Ki67>20% (+)	54	41/13	75.93		
NapsinA(+)/TTF-1(+)/Ki67>20% (–)	0	0/0	0		
NapsinA(+)/TTF-1(–)/Ki67>20% (+)	3	2/1	66.67		
NapsinA(–)/TTF-1(+)/Ki67>20% (+)	2	1/1	50.00		
NapsinA(–)/TTF-1(–)/Ki67>20% (+)	1	0/1	0		
NapsinA(+)/TTF-1(–)/Ki67<20% (–)	0	0/0	0		
NapsinA(–)/TTF-1(+)/Ki67<20% (–)	0	0/0	0		
NapsinA(–)/TTF-1(–)/Ki67<20% (–)	0	0/0	0		

CTC, circulating tumor cell.

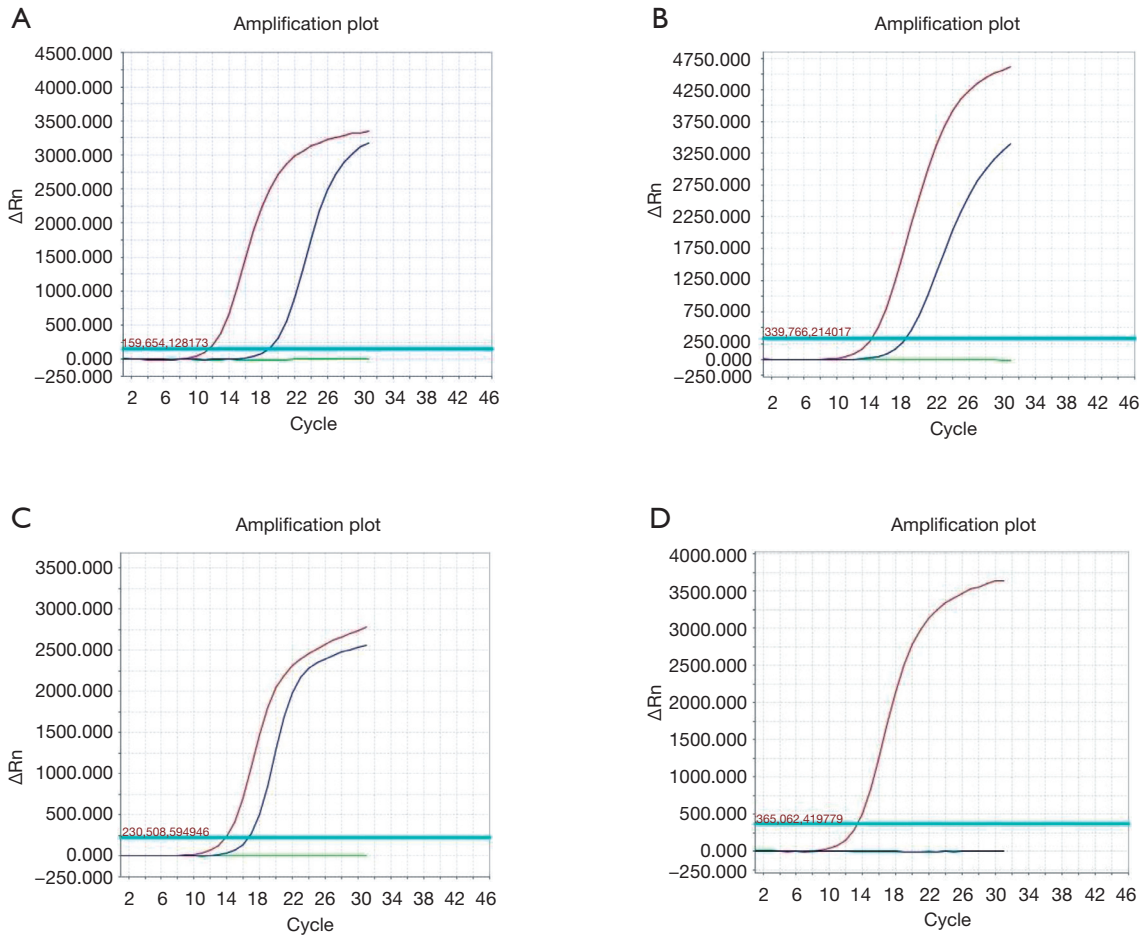

Figure 4 *EGFR* gene mutations in four exons [18, 19, 20, 21]. (A) 19-Del deletion mutation detected in exon 19 of HA-6 patient; (B) 20-ins deletion mutation detected in exon 20 of HA-22 patients; (C) L858R point mutation detected in exon 21 of HA-56 patient; (D) no mutation detected in 4 exons of HA-3 patient. The above-mentioned drawings are typical results of 60 cases of early-stage lung adenocarcinoma, and the rest are not shown in the figures.

Table 3 Circulating tumor cells and *EGFR* relationship

Clinicopathological parameters	n	Positive/negative	Positive-CTC (%)	χ^2	P value
EGFR				17.332	0.000
Mutant-type	34	32/2	94.12		
Wild-type	26	12/14	46.15		

EGFR, epidermal growth factor receptor; CTC, circulating tumor cell.

healthy or benign control groups.

Discussion

Growing evidence has established that tumor metastasis contributes to a poor clinical outcome in LCa patients (30-32). The early detection of metastasis is critical for improved therapeutic responses (33-35). Despite early diagnosis and surgical resection, most patients experience postoperative recurrence or micro-metastasis within 5 years. This compromises imaging diagnostics, and highlights the urgent need for reliable tumor markers that facilitate real-time cancer surveillance, progression, and therapeutic predictions. CTCs are cells shed from the primary tumor into the circulation. The clinical relevance of CTCs regarding tumor metastasis, prognosis, and therapeutic responses have been described for many cancers, including LCa (31,36-38). However, methods to detect LCa CTCs requires improvement in terms of sensitivity and specificity.

In this study, we isolated and characterized CTCs from PVB, but not PV, since PVB has a higher abundance of CTCs in LCa. Immunomagnetic negative selection in addition to immunofluorescence analysis provided high CTC enrichment that was highly sensitive and specific (15,16). Using this strategy, we analyzed 60 cases of early-stage LCa, and found that 73.33% of patients display positive CTC enrichment, suggesting a higher sensitivity of CTC for LCa. We therefore demonstrate that CTC detection has clinical significance for the evaluation of tumor metastasis and therapeutic responses in follow-up studies.

Examination of the correlation of CTCs with clinical parameters suggested that CTC counts were significantly associated with pathological staging and tumor infiltration, which was in accordance with previous studies (22). Higher CTC counts and positive rates were observed in patients with stage IA3 and IA2 compared to IA1 (87.50% *vs.* 77.78% *vs.* 20.00%), further indicating the role of CTCs in tumor initiation and metastasis. Additionally, for distal metastasis in IA1 stage patients, CTC counts showed improved sensitivity, whilst traditional imaging methods failed to offer useful information. A large number of clinical studies show that traditional ultrasound, CT, MRI and ECT techniques have limitations for the clinical diagnosis of malignant tumors (39). CTCs thus represent potential sources of accurate and real-time LCa detection, with lower costs, higher sensitivity, and reliability. Further studies are now necessary to develop more effective strategies for CTC detection in patients with low CTC abundances. The identified CTCs also require evaluation to confirm they originated from primary tumor tissue.

Approximately 10–35% of patients with NSCLC have tumor associated *EGFR* mutations (27). These mutations occur within *EGFR* exons 18–21, which encodes a portion of the receptor kinase domain. *EGFR* mutations are typically heterozygous, with the mutant allele also amplified (40,41). Approximately 90% of these mutations are exon 19 deletions or exon 21 L858R point mutations (42) that increase *EGFR* kinase activity leading to the hyperactivation of downstream pro-survival signaling pathways (43). More importantly, TKIs are effective in patients harboring these activating mutations. It was therefore of interest to verify whether CTC counts serve as a biomarker for the prediction of *EGFR* mutations. The results revealed a strong association ($P<0.05$) as CTCs were positive in 94.12% of patients with *EGFR* mutations, and present in 26.67% and 28.33% of patients with L858R point mutations and exon 19 deletions, respectively. Since these represent the most common *EGFR* mutations in LCa, this highlights the power of CTC counts as prognostic markers in LCa patients.

The CD45-FISH-based strategy showed clinical value as a surrogate marker for therapeutic selection and monitoring in LCa. This approach is also beneficial for the putative isolation and verification of CTCs in other cancers. Despite these benefits, future studies should focus on the development of improved isolation methods with higher sensitivity and specificity, including chromosome enumeration probes, and tumor-specific antigens for the immunofluorescent analysis of CTCs.

Our findings strengthen the clinical relevance of CTCs as prognosis, tumor metastasis and recurrence markers (44-46). However, some limitations should be noted. The sample size was small and could be extended for further validations in future studies. In addition, more accurate and solid evidence of CTCs in LCa that integrate diagnosis, predictions, and the real-time surveillance of tumor progression and therapeutic responses are required. Large-scale, multicenter follow-up studies are required to fully explore the clinical value of CTCs as predictors of LCa prognosis, recurrence and metastasis. Such studies will lay the foundation for the rationalization and individualized treatment of LCa in the clinic.

Table 4 Circulating tumor cells and *EGFR* mutation relationship

Sample	Positive/negative-CTC	Mutant/wild type-*EGFR*	Exon 18	Exon 19	Exon 20	Exon 21
HA-1	Negative	Mutant-type	–	–	–	L858R
HA-2	Positive	Mutant-type	–	19– del	–	–
HA-3	Negative	Wild-type	–	–	–	–
HA-4	Positive	Wild-type	–	–	–	–
HA-5	Negative	Wild-type	–	–	–	–
HA-6	Positive	Wild-type	–	19– del	–	–
HA-7	Positive	Mutant-type	–	19– del	–	–
HA-8	Positive	Wild-type	–	–	–	–
HA-9	Positive	Mutant-type	–	–	–	L858R
HA-10	Positive	Mutant-type	–	–	–	L858R
HA-11	Negative	Wild-type	–	–	–	–
HA-12	Positive	Mutant-type	–	–	–	L858R
HA-13	Positive	Wild-type	–	–	–	–
HA-14	Positive	Mutant-type	–	19– del	–	–
HA-15	Positive	Wild-type	–	–	–	–
HA-16	Positive	Mutant-type	–	–	–	L858R
HA-17	Positive	Mutant-type	–	19-del	–	–
HA-18	Negative	Wild-type	–	–	–	–
HA-19	Positive	Mutant-type	–	–	–	L858R
HA-20	Positive	Mutant-type	–	19-del	–	–
HA-21	Positive	Wild-type	–	–	–	–
HA-22	Negative	Mutant-type	–	–	20-ins	–
HA-23	Negative	Wild-type	–	–	–	–
HA-24	Negative	Wild-type	–	–	–	–
HA-25	Positive	Mutant-type	–	–	–	L858R
HA-26	Negative	Wild-type	–	–	–	–
HA-27	Positive	Mutant-type	–	19-del	–	–
HA-28	Positive	Mutant-type	–	–	–	L858R
HA-29	Positive	Mutant-type	–	19-del	–	–
HA-30	Positive	Mutant-type	–	19-del	–	–
HA-31	Positive	Mutant-type	–	–	–	L858R
HA-32	Negative	Wild-type	–	–	–	–
HA-33	Positive	Mutant-type	–	19-del	–	–
HA-34	Positive	Mutant-type	–	19-del	–	–
HA-35	Positive	Mutant-type	–	–	–	L858R

Table 4 (*continued*)

Table 4 (*continued*)

Sample	Positive/negative-CTC	Mutant/wild type-EGFR	Exon 18	Exon 19	Exon 20	Exon 21
HA-36	Positive	Wild-type	–	–	–	–
HA-37	Positive	Mutant-type	–	19-del	–	–
HA-38	Negative	Wild-type	–	–	–	–
HA-39	Positive	Mutant-type	–	–	–	L858R
HA-40	Negative	Wild-type	–	–	–	–
HA-41	Positive	Mutant-type	–	–	–	L858R
HA-42	Negative	Wild-type	–	–	–	–
HA-43	Positive	Mutant-type	–	19-del	–	–
HA-44	Positive	Wild-type	–	–	–	–
HA-45	Positive	Mutant-type	–	–	–	L858R
HA-46	Positive	Wild-type	–	–	–	–
HA-47	Positive	Mutant-type	–	–	–	L858R
HA-48	Positive	Wild-type	–	–	–	–
HA-49	Positive	Mutant-type	–	19-del	–	–
HA-50	Negative	Wild-type	–	–	–	–
HA-51	Positive	Wild-type	–	–	–	–
HA-52	Positive	Wild-type	–	–	–	–
HA-53	Positive	Mutant-type	–	–	–	L858R
HA-54	Negative	Wild-type	–	–	–	–
HA-55	Positive	Mutant-type	–	19-del	–	–
HA-56	Positive	Mutant-type	–	–	–	L858R
HA-57	Positive	Mutant-type	–	–	–	L858R
HA-58	Positive	Wild-type	–	–	–	–
HA-59	Negative	Wild-type	–	–	–	–
HA-60	Positive	Mutant-type	–	19-del	–	–

–, no mutation. EGFR, epidermal growth factor receptor; CTC, circulating tumor cell.

Conclusions

This study revealed that CTC counts correlate with tumor invasion, pathological staging, and *EGFR* mutations, and thus provide a promising biomarker for the surveillance of lung adenocarcinoma progression. To clarify the clinical significance of PVB-CTC status, long-term follow-up studies should now be performed.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.05.19). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all

aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Human Research Ethics Committee of the First Affiliated Hospital of University of Science and Technology of China (No. 2017-100). Written informed consent was obtained from all participants.

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