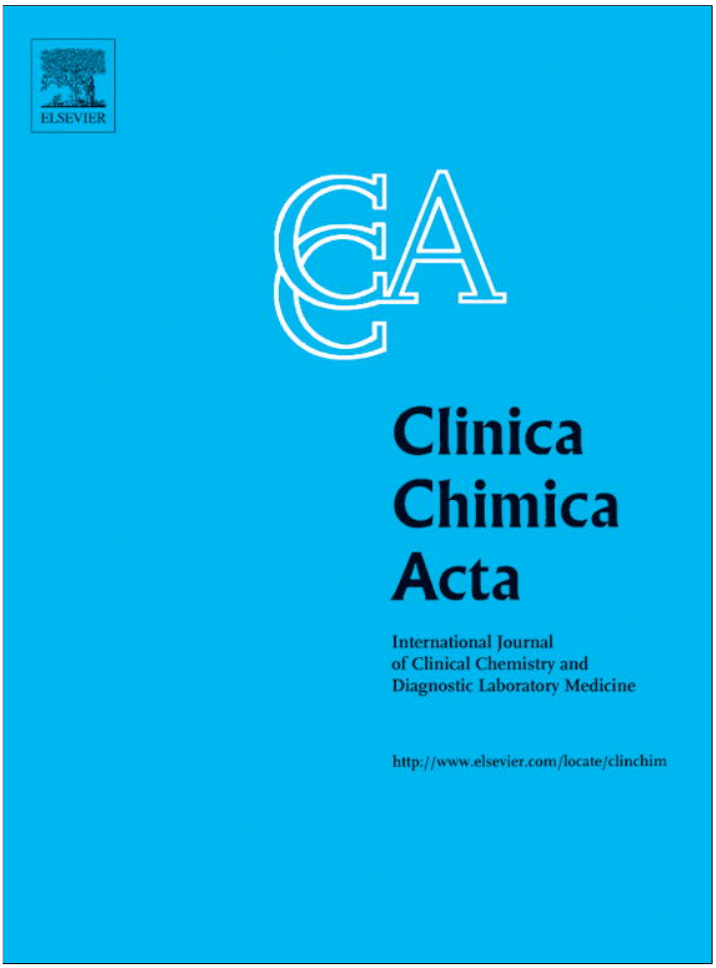

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Lung cancer circulating tumor cells isolated by the EpCAM-independent enrichment strategy correlate with Cytokeratin 19-derived CYFRA21-1 and pathological staging

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ABSTRACT

Background: Cytokeratin 19-derived CYFRA21-1 is an acceptable lung cancer biomarker. However, whether CYFRA21-1 correlates with lung cancer circulating tumor cells (CTCs) remains unclear.
Methods: CTCs in 42 lung cancer patients and 10 nonmalignant pulmonary disease patients were isolated by means of an EpCAM-independent enrichment strategy. Correlation of lung cancer CTCs with serum concentration of CYFRA21-1 and pathological staging was investigated.
Results: Among lung cancer patients in this study, 39% (7/18) of those with normal CYFRA21-1 (≤3.3 ng/ml) and 62% (13/21) of high CYFRA21-1 (>3.3 ng/ml) patients were found to have ≥3 CTCs/7.5 ml blood. The CTCs-positive rate of stage I to IV lung cancer patients was 20% (2/10), 45% (5/11), 54% (6/11) and 70% (7/10), respectively. Comparing M0 vs M1 patients, the CTCs-positive rate was 43% (13/30) and 70% (7/10), respectively. All M1 patients (10/10) had one or more CTCs detected, whereas none of the nonmalignant pulmonary disease patients had detectable CTCs.
Conclusion: Lung cancer CTCs isolated by the EpCAM-independent enrichment approach correlate with CYFRA21-1 and TNM staging. Correlation of CTCs and CYFRA21-1 in lung cancer patients is of potential clinical utility in terms of early diagnosis and predicting prognosis.

1. Introduction

Primary lung cancer is sub-classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC accounts for 20% of lung cancer cases, and NSCLC accounts for the rest 80% of cases. NSCLC can be further categorized as adenocarcinoma (ADC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC) [1]. The prognosis of lung cancer patients is generally poor, and the 5-year survival rate drops dramatically from 70% (stage I) to 15–20% (stage III) [2]. Approximately 50% of early stage patients (stages I and II) who receive resection will relapse or develop metastasis within 5 years, indicating the existence of unidentified metastatic cells at the time of resection, which cannot be detected by our current strategies.

Circulating tumor cells (CTCs) are tumor epithelial cells shed from the primary solid tumor into the blood circulation. The presence of CTCs for other cancers (breast, prostate, and colon) has had significant implications on metastasis [3,4], prognosis and the prediction of progression-free and overall survival [1,5–7]. In fact, CTC has been recently recognized by the American Society of Clinical Oncology (ASCO) as an acceptable novel tumor marker [8].

One of current strategies to isolate CTCs is epithelial cell surface molecule (EpCAM)-dependent antibody capture technology. However, due to highly heterogeneous expression [9], down-regulation [10] or even non-expression [11] of EpCAM on cancer cells derived from different tissues, the clinical application of such technique to isolate CTCs shed from different solid tumors is limited [12]. An alternative EpCAM-independent enrichment strategy is necessary.

Cytokeratins are intermediate filament structural proteins. Cytokeratin 19 (CK19) is expressed in simple epithelium, such as bronchial epithelium, and is particularly abundant in carcinoma of the lung [13,14]. Some CK19 or its fragments in tumor cells are released into the blood and circulate in the patient's circulatory system [15–17]. CYFRA21-1 is a soluble CK19 fragment. Elevated serum concentration

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of CYFRA21-1 was reported in 65–76% of NSCLC patients (stages I–IV) [18] and is now an accepted tumor marker for NSCLC [19]. Moreover, the prognostic value of CYFRA21-1 in NSCLC patients has been published elsewhere [20]. It has been reported that decreasing serum concentrations of CYFRA21-1 could predict subjects' response to chemotherapy as well as survival in patients with advanced NSCLC [21–23]. To date, it has been demonstrated that circulating CK19, the progenitor of CYFRA21-1 [24], is released from apoptotic or necrotic tumor cells [16,17,25]. Although the correlation of lung cancer CTCs with serum CK18 fragments has been observed [26,27], the relevance between lung cancer CTCs and CYFRA21-1 derived from serum CK19 remains unclear.

In the present study, we extended our previous effort [28] to further validate the EpCAM- independent enrichment strategy to detect lung cancer CTCs. Moreover, the correlation of lung cancer CTCs with serum concentration of the lung cancer biomarker CYFRA21-1 and patients' pathological staging was also investigated.

2. Materials and methods

2.1. Patients and specimens

Consent forms signed by all patients were approved by the Ethics Review Committee (ERC) of both Shi Ji Tan Hospital and PUMC Hospital. Twenty-six male and 16 female lung cancer patients with an average age of 62.2 (38–83) y were recruited for this study prior to receiving anti-cancer therapy. Among those patients, there were 6 cases of SCLC and 36 of NSCLC (14 SCC, 19 ADC, 2 LCC, and 1 carcinosarcoma) diagnosed by pathological examination. TNM staging was determined by chest radiography, bronchoscopy, brain and thoracic computed tomography (CT), positron emission tomography (PET) and bone scintigraphy according to the Union for International Cancer Control (UICC) 2009 guidelines. Seven male and 3 female patients with an average age of 66.9 (37–81) years and non-malignant pulmonary disease, including bilateral pulmonary pneumonia (n=3), bronchial asthma (n=3), acute exacerbation of chronic obstructive pulmonary disease (COPD) (n=2), acute exacerbations of chronic bronchitis (AECB) (n=1), and acute pulmonary embolism (APE) (n=1), were enrolled as controls. To avoid bias, all blood sample collection, encoding, enrichment, and identification were blindly performed by different personnel. Decoding, analysis and evaluation were co-performed by cross-blinded physicians and research scientists.

2.2. Enrichment and identification of lung cancer CTCs

Peripheral blood samples were collected via venipuncture. To avoid epithelial cell contamination, the first 2 ml of blood was collected into a tube without anti-coagulant for serum tumor marker analysis. An additional 7.5 ml of blood was subsequently collected in a tube containing ACD anti-coagulant as described previously [28]. Subjects should not have received any invasive procedures at least a week before blood was collected.

The strategy of enrichment and identification of lung cancer CTCs was essentially similar to one that we previously published [28], with minor modifications. Briefly, 7.5 ml of collected patient blood were washed with PBS, followed by lysis of RBC. The reaction mixture was centrifuged at 300 ×*g* for 5 min. The cell pellet was resuspended in PBS and subsequently incubated with anti-CD45 monoclonal antibody-coated magnetic beads for 30 min, followed by the separation of magnetic beads using a magnetic stand (Promega, Madison, WI). Supernatants were centrifuged at 500 ×*g* for 3 min. The cell pellet was resuspended in PBS and subsequently subjected to immunofluorescence analysis. The identification of enriched lung cancer CTCs was performed by co-immunostaining with monoclonal antibody anti-human cytokeratin 18 conjugated to Alexa Flour 488 (Life Technologies, CA) and monoclonal antibody anti-human CD45 conjugated to Alexa Flour 594 (Life Technologies). Cell nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI) (Life Technologies) [29,30].

2.3. Measurement of serum concentration of tumor marker proteins

Serum was separated from 2 ml of the coagulated blood sample via centrifugation (1500 *g*) at room temperature for 10 min, followed by subjection to an automatic clinical immunochemistry analyzer (Model: Cobas e601, Roche, Indianapolis, IN) for the analysis of serum tumor marker proteins, including CYFRA21-1, neuron-specific enolase (NSE), CA19-9, CA-125, CA15-3, carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP).

2.4. Statistical analysis

Statistical analysis was performed with SPSS 11.5 software (Chicago, IL). Positive rate comparison was performed by Fisher's exact test. All graphs were plotted by means of the GraphPad Prism, ver 5.0 (San Diego, CA). A 2-sided $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Identification of lung cancer CTCs enriched from patients

Lung cancer CTCs enriched from patients were subjected to immunofluorescence staining performed with Alexa Flour 488 conjugated anti-CK18 and Alexa Flour 594 conjugated anti-CD45. Cell nuclei were stained with DAPI. CTCs were defined as positive staining for DAPI and CK18 but negative for CD45 (Fig. 1). CTCs showed good morphology, as observed in a bright field.

3.2. CTCs in lung cancer patients

Ten non-malignant pulmonary disease patients and 42 lung cancer patients, including 10 with stage I, 11 with stage II, 11 with stage III and 10 with stage IV, were enrolled. Among those lung cancer patients, there were 6 SCLC and 36 NSCLC subjects. The distribution of lung cancer CTCs is shown in Fig. 2. No CTC was found in all non-malignant pulmonary disease patients. However, CTCs could be detected in the majority of lung cancer patients. The median of CTC number (CTCmed) for patients of each stage was 2 (stage I), 2 (stage II), 3 (stage III) and 4 (stage IV), respectively. Due to the small sample size in this study, unless indicated, the cutoff point

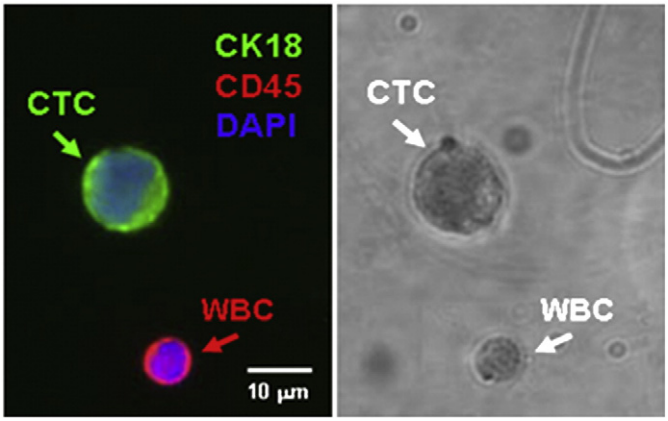

Fig. 1. Immunostaining of lung cancer CTC enriched from patient peripheral blood. CTCs enriched from lung cancer patients were subjected to immunofluorescence (IF) staining. Only CTC was specifically immunostained with anti-human CK18, but not for anti-human CD45 staining. Observed CTC showed good morphology under both immunofluorescence microscope (left) and conventional light microscope (right).

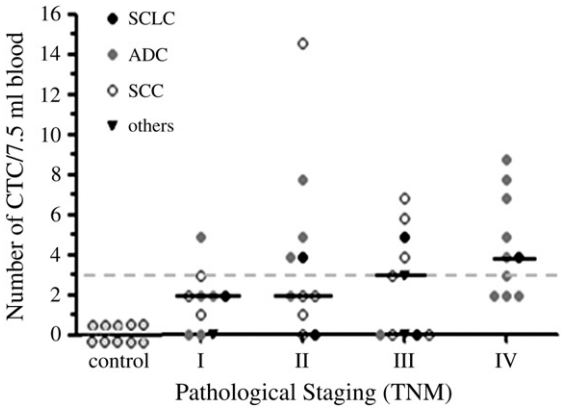

Fig. 2. Distribution of CTC in classified lung cancer and non-malignant pulmonary diseases patients. Existence of CTC in 42 lung cancer patients and a control group consisting of 10 non-malignant pulmonary diseases patients was examined. The median of CTC (CTC*med*) in those TNM staged patients was 2 (stage I), 2 (stage II), 3 (stage III) and 4 (stage IV), respectively. None of non-malignant pulmonary diseases patients had detectable CTC.

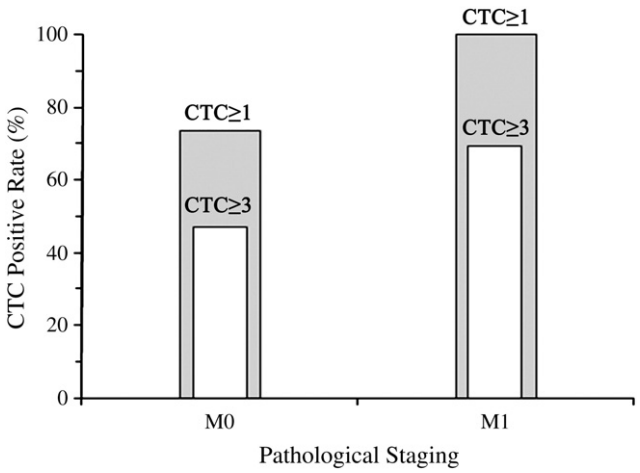

Fig. 4. Lung cancer CTCs have a relevance to staging M1. Lung cancer CTCs positive rate (CTC≥3/7.5 ml blood) of M0 patients vs M1 patients was 43% (13/30) vs 70% (7/10), whereas all of M1 patients (100%) and 73% of M0 patients were found to have at least 1 or more CTCs detected (CTCs≥1).

was defined as the average of the CTCmed of stage I - IV, which was 3 cells/7.5 ml blood.

3.3. Lung cancer CTCs correlate with TNM staging

As revealed in Fig. 3, 20% (2/10) of stage I, 45% (5/11) of stage II, 54% (6/11) of stage III and 70% (7/10) of stage IV patients were found to have CTCs≥3/7.5 ml blood. To further understand if tumor size, invasiveness, lymphatic and distant metastasis have an impact on CTCs counting, additional statistical analysis was performed on different categories of TNM. As shown in Fig. 3, following an increase in patients' tumor size from T1 to T4, 33% (3/9) of T1, 46% (6/13) of T2, 63% (5/8) of T3, and 60% (6/10) of T4 patients had CTCs≥3.

With respect to the correlation of CTCs with distant metastasis, the results of Fig. 4 showed that 73% of M0 and all (100%) M1 patients had at least 1 or more detectable CTCs (CTCs≥1), and 43% (13/30) of M0 and 70% (7/10)of M1 patients had at least 3 or more CTCs detected (CTCs≥3).

Additional analysis on different cancer subtypes of those M0 patients indicated that the CTCs-positive rate (CTCs≥3) of SCLC, ADC and SCC patients was similar, which was 50%, 40% and 46%, respectively. However, among those M1 patients, only ADC had the highest CTCs positive rate of approximately 70% (data not shown).

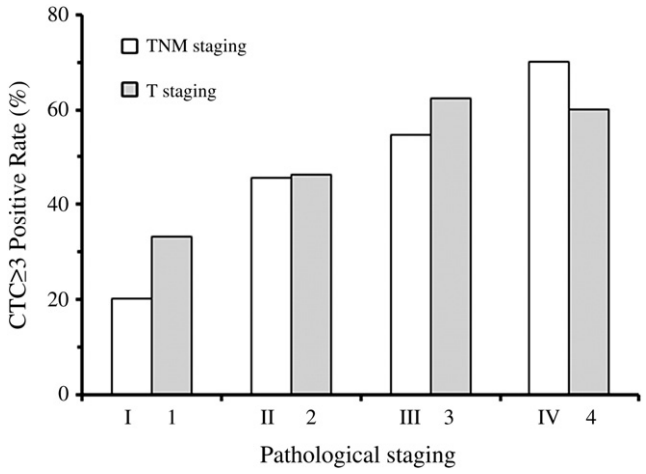

Fig. 3. Lung cancer CTCs correlates with TNM staging. Lung cancer CTCs positive rate (CTCs≥3/7.5 ml blood) increased in advanced stage patients. It was 20% (2/10) with TNM stage I, 45% (5/11) with stage II, 54% (6/11) with stage III, and 70% (7/10) with stage IV. Similar observation was achieved in patients staged by T. It was 33% (3/9) with T1, 46% (6/13) with T2, 63% (5/8) with T3, and 60% (6/10) with T4.

3.4. Lung cancer CTCs correlate with CYFRA21-1

CTCs enumeration was performed on 18 patients who had a normal serum concentration of CYFRA21-1 (≤3.3 ng/ml) and 21 patients who had a high CYFRA21-1 (>3.3 ng/ml). Fig. 5A showed that 39% (7/18) of patients who had normal CYFRA21-1 were found to have more than 3 CTCs, whereas 62% (13/21) of high CYFRA21-1 patients had 3 or more detectable CTCs. Further analysis on the same population of patients indicated that 61% (11/18) of normal and 95% (20/21) of high CYFRA21-1 patients had at least 1 or more CTCs detected, the difference of 61% vs 95% was statistically significant ($P=0.015$). No obvious significant correlation between lung cancer CTCs and other serum tumor markers, including NSE, CA19-9, CA-125, CA15-3, CEA and AFP, was found (data not shown) in the current small sample size.

Further statistical analysis was performed to investigate whether there was any correlation among CTCs counting, TNM staging and serum concentration of CYFRA21-1. Revealed in Fig. 5B, in those who had a normal concentration of CYFRA21-1, more than 3 CTCs could be detected in 20% of TNM stage I (1/5), 40% of stage II (2/5), 0% of stage III (0/3), and 80% of stage IV (4/5) patients. In the group of high CYFRA21-1 patients, the CTCs positive rate (CTCs≥3) was 33% (1/3) for stage I, 50% (3/6) for stage II, 86%(6/7)for stage III and 60% (3/5) for stage IV, respectively.

4. Discussion

EpCAM is the epithelial cell surface adhesion molecule. Direct capture of CTCs using anti-EpCAM antibody has been reported elsewhere [6,31]. However, expression of EpCAM on the surface of epithelial tumor cells is highly heterogeneous, and some types of cancer cells even do not express EpCAM [11,32,33]. Moreover, inevitable down regulation of EpCAM in cancer cells is believed to be part of an oncogenic pathway that allows epithelial tumor cells to dissociate from the primary tumor and to increase tumor invasiveness and metastasis [10,12]. Due to those inherent disadvantages of EpCAM-based technology, an EpCAM-independent enrichment approach becomes necessary. Instead of attempting to capture CTCs through antibody binding to EpCAM, the enrichment strategy depletes irrelevant hematopoietic cells and undesired components in blood, resulting in the enrichment of circulating rare cells, including

epithelial and endothelial cells. In the current study, we continued our previous efforts [28] to further validate our enrichment technology and to investigate the clinical significance of lung cancer CTCs. Reproducible performance of such an EpCAM-independent enrichment approach with respect to isolating lung cancer CTCs was confirmed in this study.

Correlation of CTCs and TNM staging was further investigated. As shown in Fig. 2, only 20% of stage I patients had CTCs≥3, whereas 45% of stage II, 54% of stage III and 70% of stage IV patients had CTCs≥3. None of non-malignant pulmonary disease patients had any CTC detected. Statistical analysis of T1–T4 staging indicated that the detection rate of CTCs≥3 increased from 41%(9/22) of T1–2 patients to 61%(11/18) of T3–4 patients, indicating that CTCs number is proportional to increased invasiveness. The results obtained from the analysis of M0 and M1 patients clearly showed that among M1 patients who had distant metastasis, all of them had one or more CTCs detected. Among M0 patients who did not have distant metastasis diagnosed by means of a clinical imaging system, 73% of patients had at least 1 or more detectable CTCs (CTCs≥1), suggesting that detecting CTCs is more sensitive than conventional CT with respect to monitoring lung cancer patients' distant metastasis. None of the nonmalignant pulmonary disease patients had detectable CTCs.

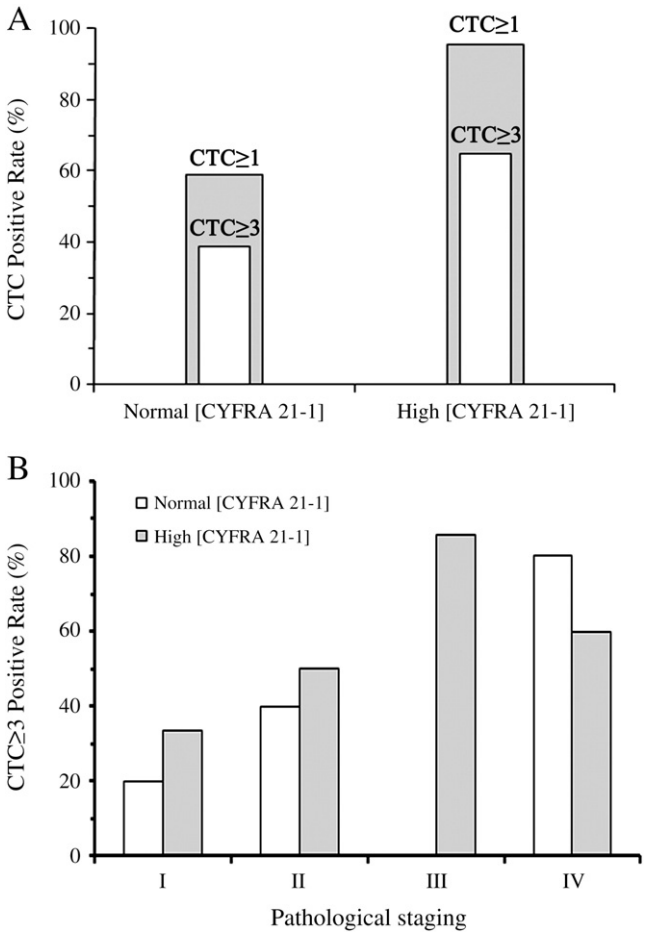

Fig. 5. Lung cancer CTCs correlates with CYFRA21-1. CTC enumeration was analyzed in patients with normal and high serum concentration of CYFRA21-1. (A) 39% of normal CYFRA21-1 lung cancer patients had CTCs≥3 compared to the rate of 62% with high CYFRA21-1 patients. 61% of normal concentration, and 95% of high CYFRA21-1 patients had at least 1 or more CTCs detected. The difference was statistically significant ($P=0.015$). (B) In the group of normal CYFRA21-1 patients, CTCs positive rate (CTCs≥3) was 20% for TNM stage I, 40% for stage II, 0% for stage III, and 80% for stage IV. To those patients with high CYFRA21-1, the positive rate (CTCs≥3) was 33% for stage I, 50% for stage II, 86% for stage III, and 60% for stage IV.

In view of the obvious clinical significance, CTC has been recently recommended by the ASCO as a potentially acceptable tumor marker [8]. In this study, we intensively investigated the relevance between CTCs counting and any one of a series of tumor markers, including NSE, CA19-9, CA-125, CA15-3, CEA, AFP and the lung cancer marker CYFRA21-1. The results obtained in this small sample size study indicated that CTCs correlated only with CYFRA21-1, more CTCs counts were detected in patients with high serum concentration of CFRA21-1 than in those with normal CFRA21-1.

To date, it has been realized that circulating cytokeratins (CKs) released from apoptotic tumor cells into blood are the significant markers for evaluation of therapeutic efficacy as well as monitoring tumor relapse [15]. Currently, three cytokeratin-derived markers – CK18-derived tissue polypeptide specific antigen (TPS) [26,27], CK8, 18, 19-derived tissue polypeptide antigen (TPA), and CK19-derived CYFRA21-1 – have been accepted as tumor markers. CK19 localizes in the bronchial epithelium and is abundant in lung cancer cells [13,14]. In apoptotic cancer cells of primary tumors, CK19 is subjected to caspase 3 proteolytic processing, resulting in the release of CYFRA21-1 into peripheral blood [16,17,34]. Study results recently published by others indicated that serum CYFRA21-1 in NSCLC patients had a significant correlation with both radiological response and prognosis [23]. In this study, we demonstrated for the first time that CTCs correlate with CYFRA21-1. Interestingly, the correlation of apoptotic CTCs and CK18-derived TPS in SCLC patients has been recently reported [26,27]. It is reasonable to speculate that the apoptosis of some lung cancer CTCs triggers the intracellular proteolysis of CK19, resulting in the generation and release of CYFRA21-1 into peripheral blood. This might help to explain how lung cancer CTCs correlate with CYFRA21-1.

The obtained results illustrate the promising clinical utility of lung cancer CTCs. However, the sample size of this preliminary study was small, and it is necessary to perform a large-scale study to further understand the clinical significance of lung cancer CTCs and its correlation to CYFRA21-1 and other tumor serum markers.

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