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Original Article

Folate receptor-positive circulating tumor cells in predicting ground-glass nodules malignancy

Yi-Sen Yue[1*], Zhao-Hui Sun[2,3*], Qian Cui[4], Guo-Zhong Jiang[5]

[1]College of Basic Medicine and Forensic Medicine, Henan University of Science and Technology, Luoyang, Henan, P. R. China; [2]Department of Laboratory Medicine, The General Hospital of Southern Theatre Command of PLA, Guangzhou, Guangdong, P. R. China; [3]The First School of Clinical Medicine, Southern Medical University, Guangzhou, Guangdong, P. R. China; [4]Department of Pathology, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, Guangdong, P. R. China; [5]Department of Pathology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, P. R. China. [*]Equal contributors and co-first authors.

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Abstract: Objective: To study the clinical significance of folate receptor-positive circulating tumor cells (FR+CTCs) in determining malignancy of ground-glass nodules (GGNs) and assess the added value of FR+CTC in the classic GGN evaluation model (Mayo Model). Methods: Sixty-five patients with single indeterminate GGN were recruited. Twenty-two participants had benign/pre-malignant diseases, and forty-three had lung cancers, as confirmed by histopathology examination. FR+CTC was enumerated by CytoploRare® Kit. A CTC model was drawn based on the multivariate logistic analysis. The area under the receiver operating characteristic curve (AUC) was analyzed to evaluate the diagnostic performance of FR+CTC, CTC model and Mayo model. Results: In the cohort, the mean age of 13 males and 9 females with benign/pre-malignant diseases was 57.7 ± 10.2 years. The mean age of 13 males and 30 females with lung cancers was 53.8 ± 11.7 years. There was no significant difference between the age and the smoking history (P=0.196 and P=0.847, respectively). FR+CTC can effectively differentiate lung cancer from benign/pre-malignant diseases [sensitivity: 88.4%, specificity: 81.8%, the AUCs was 0.8975, 95% confidence interval (CI): 0.8174-0.9775] in patients with GGN. Multivariate analysis revealed that FR+CTC level, tumor size, and tumor location were independent predictors of GGN malignancy (P<0.05). The prediction model based on these factors showed better diagnostic efficiency than the Mayo model (AUC: 0.9345 vs. 0.6823), yielding superior sensitivity (81.4% vs. 53.5%) and specificity (95.5% vs. 86.4%). Conclusion: The FR+CTC exhibited a promising potential in determining the malignancy of indeterminate GGNs, and the CTC model's diagnostic efficiency was superior to the Mayo model.

Keywords: Circulating tumor cell, folate receptor, ground-glass nodule, malignancy, prediction model

Introduction

Since the launch of the National Lung Screening Trial (NLST) study, computed tomography (CT) has been widely adopted in lung cancer screening worldwide, which has led to a significant increase in the number of indeterminate pulmonary nodules identified [1]. The differential diagnosis of these identified nodules is now a global clinical challenge, especially for ground-glass opacity nodules (GGNs), due partly to a lack of clear structural characteristics. A GGN refers to a hazy, opaque nodule that does not obscure the underlying bronchial structures and pulmonary vessels. While it may implicate lung cancer, a GGN can also be caused by other non-malignant conditions such as fibrosis, inflammatory diseases, and neoplasms [2]. The probability of malignancy in GGNs varies greatly among studies, ranging from 27% to 63% [3, 4]. Currently, there is a lack of consensus on the management of GGNs, and follow-up surveillance is usually recommended for a GGN ≥6 mm [5]. An enlargement of the solid components at a follow-up generally indicates malignancy, while nodule shrinkage at follow-up or after antibiotics treatment implies benignancy. Unfortunately, as GGNs usually have a stable appearance, radiological surveillance of these nodules may take months to years, which leads

to a delay in the timely treatment of patients with malignant diseases [6]. In practice, the decision and the timing to perform surgical treatment are mainly based on a physician's personal experiences. Tumor biomarkers, such as carcinoembryonic antigen (CEA), neuron-specific enolase (NSE), squamous cell carcinoma (SCC) and cytokeratin fragment 19 (CYFRA21-1), may assist in determining malignancy, but their sensitivity and specificity in early-stage lung cancer are dismal [7, 8]. Tissue biopsy remains the only means to confirm a malignancy, but it is invasive and practically infeasible in some patients. Therefore, developing a novel, non-invasive biomarker is essential to improve the differential diagnosis of GGNs.

The estimation of GGN malignancy has long been a focus area strongly related to the early diagnosis and management of lung cancer. The Mayo model is a classic mathematical model for predicting malignancy of pulmonary nodules via synthetic analysis of different clinical and CT characters. Its successors, such as the Veterans Affairs model and the Brock model, have similar calculation formulas, so we selected the original model to make a comparison [9, 10]. Circulating tumor cell (CTC) is a tumor biomarker in peripheral blood, even at the early stage of cancer [11]. CTC has also been proven a strong prognostic factor for several types of cancer [12-15]. To date, limited studies are exploring the performance of CTC in differential diagnosis or screening of early-stage cancers. For lung cancer, conventional EpCAM-based CTC detection techniques, such as the CellSearch system, suffer from low sensitivity [16]. In 2016, the detection of folate receptor-positive CTC (FR+CTC) based on the ligand-targeted polymerase chain reaction method was approved by the National Medical Products Administration (NMPA) for quantitative detection of FR+CTC in human whole blood in vitro. Previous studies have reported superior diagnostic efficiency of FR+CTC in diagnosing lung cancer with different pathological subtypes and stages [17-20]. In addition, FR+CTC levels were associated with chemotherapy response and prognosis in non-small cell lung cancer [21-23]. Currently, there are no systematic studies investigating the clinical significance of FR+CTC in the management of GGNs. Hence, the present study sought to establish a novel prediction model using FR+CTC to assist in the differential diagnosis of GGNs.

Methods

Study design

This is a preliminary, prospective, observational study to explore the significance of FR+CTC in determining the malignancy of GGNs. A total of 65 patients with a single indeterminate GGN were recruited from July 2018 to September 2018 at Guangdong Provincial People's Hospital and The First Affiliated Hospital of Zhengzhou University. A chest CT scan was performed on all the participants. Only treatment-naive patients with indeterminate GGNs were recruited in this study. A GGN was defined as any nodules identified with ground-glass opacity component (either pure GGNs or mixed GGNs). Patients without a clear pathological examination or sufficient preoperative blood samples collected for FR+CTC analysis and those whose blood samples had hemolysis or coagulation were excluded. Tumor samples were assessed by at least two independent experienced pathologists for pathological subtyping and staging according to the AJCC Cancer Staging Manual (8th edition, 2017). Based on the pathological results, 22 patients with benign or premalignant diseases and 43 patients with lung cancers were included. The ethics committee of each participating hospital has approved the study protocol prior to the initiation of patient enrollment (2023-KY-0199). The study was conducted according to the Declaration of Helsinki. Written informed consent was obtained from each participant.

FR+CTC analysis

Before starting any treatment, 3 mL of peripheral venous blood from each participant was collected using a 6 mL EDTA-containing tube for FR+CTC analysis. FR+CTC level was analyzed using the CytoploRare® Kit (Genosaber Biotech, Shanghai, China) within 24 hours after blood sample collection. The analysis was performed according to the manufacturer's instructions. Briefly, the negative enrichment method was used to deplete erythrocytes and leukocytes. The enriched samples were then treated with a "ligand-targeted polymerase chain reaction" to enumerate FR+CTC. A proprietary detection probe consisting of a folate receptor alpha-targeting folate ligand coupled with a special oligonucleotide was used to label the FR+CTCs [24]. A conventional polymerase chain

Table 1. Patients' characteristics

	Benign/pre-malignant diseases (n=22)	Lung cancers (n=43)	*P* value
FR+CTC level	6.5 (1.7-12.1)	10.0 (3.3-25.0)	<0.001
Gender			0.025
Male	13 (59)	13 (30)	
Female	9 (41)	30 (70)	
Age	57.7 ± 10.2	53.8 ± 11.7	0.196
Tumor size	1.26 ± 0.70	1.90 ± 1.12	0.018
Location			<0.001
Upper lobe	6 (27)	31 (72)	
Middle or lower lobe	16 (73)	12 (28)	
Smoking history			0.847
Ever	4 (18)	7 (16)	
Never	18 (82)	36 (84)	
Pathological stage			
I	/	34 (79)	
II	/	1 (2)	
III	/	2 (5)	
IV	/	4 (9)	
Uncertain	/	2 (5)	

Folate receptor-positive circulating tumor cell (FR+CTC) levels were expressed as median (range) and measured in FU/3 mL. The gender, location, smoking history, and pathological stage were expressed as number (percentage). Age was expressed as mean ± standard deviation and measured in years. Tumor size was expressed as mean ± standard deviation and measured in cm.

reaction was performed to quantify the signal of the special oligonucleotide attached to FR+CTCs. Folate receptor Unit (FU), as defined in the manufacturer's manual, was used to represent the level of FR+CTC in 3 mL of peripheral blood.

Prediction models

The Mayo model is defined as follows. The probability of malignancy = $e^{\alpha}/(1 + e^{\alpha})$, where α = -6.8272 + (0.0391 × age) + (0.7917 × smoking history) + (1.3388 × cancer history) + (0.1274 × tumor size) + (1.0407 × spiculation) + (0.7838 × location). In the above model, "1" stands for "yes" and "0" stands for "no" for smoking history, cancer history, and spiculation. For the location, "1" stands for the upper lobe and "0" stands for the middle or lower lobe. Age is measured in years. Tumor size is the maximum nodule diameter measured in mm, as observed in the CT scan. The CTC-based prediction model was established using multiple logistic regression analysis based on the likelihood ratio test (forward stepwise method). Clinical and radiological factors included in the model were gender, age, tumor size, location, smoking history, and FR+CTC level.

Statistical analysis

Statistical analyses were performed using SPSS 22.0 (SPSS Inc., IL, USA) and Prism 8 (GraphPad Software Inc., CA, USA). Categorical variables were presented in counts (percentages). FR+CTC level was presented in median (range). Age and tumor size were presented in mean ± standard deviation. The Chi-square test was used to compare the difference in categorical variables between pathological subgroups. For continuous variables, the Mann-Whitney test was used for FR+CTC, and the two-sample t-test was used for age and tumor size. The receiver operating characteristic (ROC) curve was used to examine the diagnostic efficiency of FR+CTC and the prediction models in determining malignancy. The optimal cutoff value was decided based on the Youden index, calculated as sensitivity + specificity - 1. Independent predictors of malignancy were determined by multivariate analysis using logistic regression.

Results

Patients' characteristics

Of the 65 recruited patients with single indeterminate GGNs, 18 patients were pathologically diagnosed with benign diseases (including 7 with inflammatory diseases and 11 with interstitial lung diseases), 4 patients had pre-malignant diseases (including 2 with atypical adenomatous hyperplasia and 2 with adenocarcinoma *in situ*), and 43 patients had lung cancers (including 33 with adenocarcinomas, 8 with non-small cell lung cancer not otherwise specified, 1 with squamous cell carcinoma, and 1 with small cell lung cancer). Detailed clinical characteristics are shown in **Table 1**. The FR+CTC level and tumor size of lung cancer

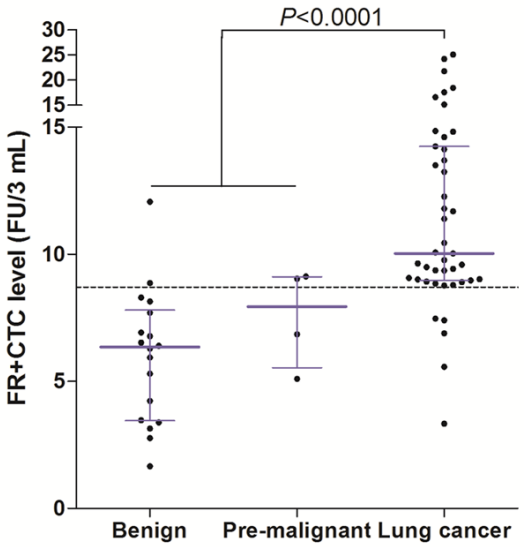

Figure 1. The FR+CTC level according to pathological subtype. The dot plot compares the folate receptor-positive circulating tumor cell (FR+CTC) level between patients with benign diseases (n=18), pre-malignant diseases (n=4), and lung cancers (n=43). Each dot represents an individual patient. Lines indicate median and interquartile range. The dotted line at 8.7 FU/3 mL was the optimal cutoff value determined. FU represents the "folate receptor unit".

patients were significantly higher/larger than those with benign or pre-malignant diseases (P<0.01, P=0.018, respectively). In addition, upper lobe nodules appeared to be associated with lung cancer more often than middle/low lobe nodules (P<0.01). Gender was significant in the model (P=0.025), but such an association may be confounded by the fact that most of patients were non-smokers and that the sample size was small. There were no significant differences in age or smoking history between the two patient groups (P=0.196, P=0.847, respectively).

ROC analysis

As shown in **Figure 1**, lung cancer patients had a significantly higher FR+CTC level (median: 10.0 FU/3 mL, ranging from 3.3 FU/3 mL to 25.0 FU/3 mL) compared to those with benign diseases (median: 6.4 FU/3 mL, ranging from 1.7 FU/3 mL to 12.1 FU/3 mL) and those with pre-malignant diseases (median: 7.9 FU/3 mL, ranging from 5.1 FU/3 mL to 9.1 FU/3 mL) (P<0.01). Based on the Youden index, 8.7 FU/3 mL was determined as the optimal cutoff value for FR+CTC, consistent with the manufacturer's manual.

In a ROC analysis, FR+CTC level showed high sensitivity and specificity (sensitivity: 88.4%, specificity: 81.8%, 95% CI: 0.8174-0.9775, P<0.01) in differentiating lung cancers from benign or pre-malignant diseases in patients with indeterminate GGNs (**Table 2**). The performances of FR+CTC in differentiating benign diseases from pre-malignant diseases and in differentiating pre-malignant diseases from lung cancers were slightly lower (**Figure 2A**). However, even though the AUC of FR+CTC in differentiating benign diseases from pre-malignant diseases was 0.722 (95% CI: 0.4409-1.004, P=0.173), there is no significant difference between these two groups (**Table 2**).

Multivariate analysis

Multiple logistic regression analysis was conducted to assess the predictive value of different clinical factors in determining the malignancy of GGNs, and a likelihood ratio test was applied. As shown in **Table 3**, FR+CTC level, tumor size, and location were the only factors that could independently predict malignancy (P<0.05). Based on the logistic regression analysis, we established a prediction model, the CTC model, for determining the malignancy of GGNs. The model is as follows. The probability of malignancy = $e^{\alpha}/(1 + e^{\alpha})$, where α = -8.016 + (0.671 × FR+CTC level) + (1.029 × tumor size) + (2.334 × location). FR+CTC level is measured in FU/3 mL. Tumor size is the maximum nodule diameter measured in cm, as observed in the CT scan. For the location, "1" stands for the nodule located at the upper lobe and "0" stands for the nodule located at the middle or lower lobe.

Comparing the CTC model with the Mayo model

ROC analysis was used to compare the diagnostic efficiency of the newly established CTC model with the existing Mayo model. Results showed that the CTC model has better diagnostic efficiency than the Mayo model in terms of AUCs (0.9345, 95% CI: 0.8758-0.9931 vs. 0.6823, 95% CI: 0.5526-0.8121; **Table 4**). Meanwhile, "FR+CTC alone" provides a diagnostic efficiency between the CTC model and

Table 2. Receiver operating characteristic analysis of FR+CTC

	Benign diseases vs. Pre-malignant diseases	Pre-malignant diseases vs. Lung cancers	Benign diseases and Pre-malignant diseases vs. Lung cancers
Area under curve	0.7222	0.8256	0.8975
95% confidence interval	0.4409-1.004	0.6631-0.9881	0.8174-0.9775
P value	0.173	0.033	<0.001
Cutoff value	9.0	9.2	8.7
Sensitivity	50.0%	67.4%	88.4%
Specificity	94.4%	100%	81.8%

FR+CTC represents folate receptor-positive circulating tumor cell.

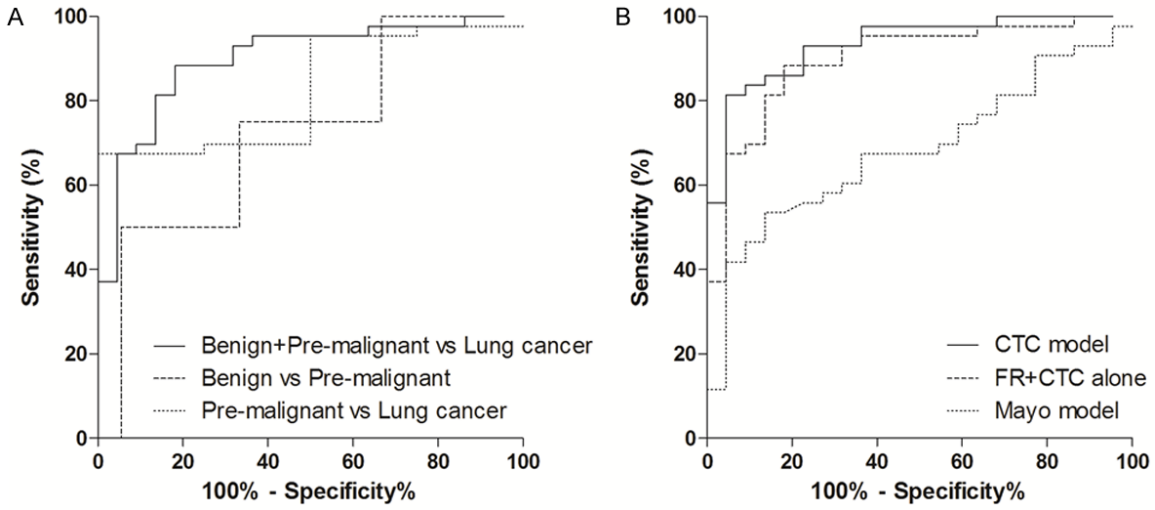

Figure 2. Receiver operating characteristic analysis. A. The receiver operating characteristic curves of folate receptor-positive circulating tumor cell (FR+CTC) in differentiating benign diseases and pre-malignant diseases (n=22) from lung cancers (n=43), benign diseases (n=18) from pre-malignant diseases (n=4), and pre-malignant diseases (n=4) from lung cancers (n=43). B. The receiver operating characteristic curves of the CTC model, FR+CTC alone, and the Mayo model in differentiating benign diseases and pre-malignant diseases (n=22) from lung cancers (n=43).

Table 3. Multivariate analysis in determining malignancy

	B	S.E	Wals	Odds ratio	95% confidence interval	*P* value
FR+CTC	0.671	0.195	11.808	1.956	1.334-2.867	0.001
Tumor size	1.029	0.483	4.540	2.798	1.086-7.208	0.033
Location	2.334	0.912	6.549	10.322	1.727-61.689	0.010
Constant	-8.016	2.200	13.270	/	/	/

FR+CTC represents folate receptor-positive circulating tumor cell.

Table 4. Receiver operating characteristic analysis of the prediction models

	Mayo model	FR+CTC alone	CTC model
Area under curve	0.6823	0.8975	0.9345
95% confidence interval	0.5526-0.8121	0.8174-0.9775	0.8758-0.9931
P value	0.017	<0.001	<0.001
Cutoff value	/	8.7	/
Sensitivity	53.5%	88.4%	81.4%
Specificity	86.4%	81.8%	95.5%

FR+CTC represents folate receptor-positive circulating tumor cell.

the Mayo model (AUC: 0.8975, 95% CI: 0.8174-0.9775; **Figure 2B**).

Discussion

Lung cancer screening can potentially identify early-stage lung cancers that are still curable. However, current practices still need to improve on risks of false negative/positive results, radiation exposure, unnecessary distress, and complicated follow-up processes [25]. Prediction models such as the Mayo model have been established to enhance the performance of the differential diagnosis of pulmonary nodules. The Mayo model demonstrated adequate diagnostic efficiency through a comprehensive analysis of the clinical and imaging features. However, its sensitivity and specificity were lower in the Asian population [26], probably due to the unique characteristics of the region, such as high levels of air pollution and higher prevalence of several infectious diseases [27].

Our study implied that FR+CTC consistently performed well in the differential diagnosis of pulmonary GGNs malignancy. The sensitivity and specificity of FR+CTC were as high as 88.4% and 81.8% in differentiating lung cancers from benign or pre-malignant diseases. Our study is the first to apply FR+CTC in detecting indeterminate GGNs with satisfactory diagnostic performance. Previous studies mainly focused on proving that FR+CTC is an efficacious biomarker in lung cancer diagnosis [15-18]. Furthermore, our results, including the FR+CTC level, the optimal cutoff value, sensitivity, and specificity were similar to those of the previous studies [29, 30], suggesting that the FR+CTC test was robust and applicable to both GGNs and the general lung nodules.

Furthermore, a CTC model was established in this study, and its performance was compared to the Mayo model. With multivariate analysis, FR+CTC level, tumor size, and location were determined to be independent predictors of malignancy in GGNs, consistent with previous findings [31]. The CTC model combining these factors showed an enhanced diagnostic efficiency (sensitivity: 81.4%, specificity: 95.5%) in the differential diagnosis of GGNs. The performance of the CTC model was superior to the Mayo model (AUC: 0.9345 vs. 0.6823).

Our study has several limitations. First, the sample size was small, and no validation data set was used to examine the performance of the established CTC model. Therefore, further studies with larger sample sizes are required to validate our results. Second, clinical factors such as detailed imaging features and family cancer history were unavailable for some participants, making it impossible to compare the performance of the CTC model with other prediction models such as the PU model, one that was established using the Chinese population [32].

To conclude, the FR+CTC test showed promising performance in determining the malignancy of indeterminate GGNs, and the CTC model provided a better diagnostic efficiency than the currently available prediction model. Overall, we recommend that the FR+CTC test and the CTC model should be applied in routine clinical practice to improve the differential diagnosis of indeterminate GGNs. A further systematic study is required to validate the clinical diagnostic efficiency of FR+CTC and the CTC model in GGNs.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Guo-Zhong Jiang, Department of Pathology, The First Affiliated Hospital of Zhengzhou University, 1 Jianshe Road, Zhengzhou, Henan, P. R. China. E-mail: guozhongjiang@zzu.edu.cn

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