

RESEARCH ARTICLE

Folate receptor-positive circulating tumor cells in the preoperative diagnosis of indeterminate pulmonary nodules

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Abstract

Background: The use of FR+CTC to distinguish lung cancer from benign lung disease has been well studied. However, the effective method to differentiate precursor glandular lesions from benign/malignant pulmonary diseases is rare.

Methods: 380 patients with indeterminate pulmonary nodules were prospectively recruited. Peripheral blood samples were collected from all participants before surgery for analyzing FR+CTC levels. The performance of FR+CTC to identify lung precursor lesions were analyzed by receiver operating characteristic (ROC) curve.

Results: FR+CTC can effectively differentiate precursor from benign pulmonary diseases in all included patients (cutoff: 9.22 FU/3 ml, AUC = 0.807, ($p < 0.0001$, sensitivity: 69.17%, specificity: 82.46%) and patients with single pulmonary lesion (cutoff: 9.03 FU/3 ml, AUC = 0.842, $p = 0.0001$, sensitivity: 75.20%, specificity: 83.00%). However, FR+CTC cannot differentiate precursor from benign pulmonary diseases in multiple lesions patients ($p = 0.110$). FR+CTC neither differentiate precursor from malignant pulmonary lesions in all included patients ($p = 0.715$), single nor multiple lesions patients ($p = 0.867$, $p = 0.692$, respectively). Total number of pulmonary nodules, MTD, location (lower vs upper) were independent risk factors for malignancy (AOR, 95% CI: 3.104 (1.525, 6.316), 3.148 (1.722, 5.754), 2.098 (1.132, 3.888), respectively).

Conclusion: Preoperative FR+CTC can be identified in precursor glandular lesions and utilized to differentiate from benign pulmonary diseases. Total number of pulmonary nodules, MTD, location (lower vs upper) were independent risk factors for malignancy.

KEYWORDS

adenocarcinoma in situ, folate receptor, lung neoplasms, neoplastic cells, circulating, preoperative diagnosis

1 | INTRODUCTION

Adenocarcinoma in situ (AIS) was formerly classified as a subtype of lung adenocarcinoma that is usually considered pre-invasive.

According to the definition of the International Association for the Study of Lung Cancer (IASLC, 2011 edition), the American Thoracic Society (ATS) classification in 2011, and the European Respiratory Society (ERS) classification in 2011, AIS is a localized adenocarcinoma

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lesion of less than or equal to 3 cm with pure lepidic growth pattern.¹ In WHO classification of thoracic tumors (5th edition) 2021, AIS and atypical adenomatous hyperplasia (AAH) were adjusted from preinvasive lesions to precursor glandular lesions.²

Previous studies revealed that AIS shows distinctive clinicopathological features, genetic signatures, microenvironmental conditions, and DNA methylation levels and destined to be different from AAH, minimally invasive adenocarcinoma (MIA), and invasive adenocarcinoma (IA).³⁻⁶ More importantly, patients with AIS demonstrated superior prognosis after curative resection, with 5-year disease-specific survival (DFS) and overall survival (OS) of nearly 100%.^{7,8} Because of its noninvasive nature, AIS has been proposed to be an appropriate candidate for limited resection.⁹ Therefore, preoperative identification of AIS is of great concern. However, a reliable method is scarce. Positron emission tomography/computed tomography (PET/CT) showed limited sensitivity in identifying AIS.¹⁰ By comparison, perioperative frozen section diagnosis typically has high specificity, it shows limited sensitivity and usually overestimates the tumor invasiveness.¹¹ At the right time, circulating tumor cell (CTC) detection technology may provide insights into tumor invasiveness and help identify AIS prior to surgery. Over the past decade, different techniques have been developed to isolate, detect, and quantitate CTCs, and the clinical significance of CTC has been well studied.¹² The Cytoplorare® CTC enrichment and detection kit enumerates folate receptor-positive circulating tumor cells (FR+CTCs) using negative enrichment and ligand-targeted polymerase chain reaction (PCR) techniques. The FR+CTC level has proven effective in determining the malignancy of pulmonary nodules.¹³⁻¹⁷ Furthermore, FR+CTCs were found to be present in patients with preinvasive pulmonary diseases.^{18,19} Thus, in the present study, we aimed to investigate the performance of FR+CTC as a noninvasive biomarker to preoperatively identify lung precursor lesions.

2 | MATERIAL AND METHODS

2.1 | Patient recruitment

The observational study conducted in Shanghai Pulmonary Hospital. A total of 380 patients with indeterminate pulmonary nodules were recruited from July 2018 to June 2019. Inclusion criteria were as follows: (1) patients with indeterminate pulmonary nodules of less than or equal to 30mm according to CT scan; (2) expected to undergo surgical resection; and (3) signed informed consent and agreed to provide a preoperative peripheral blood sample for FR+CTC analysis. Exclusion criteria were as follows: (1) uncertain pathological diagnosis of surgically resected specimens; (2) insufficient or abnormal morphology preoperative blood sample for FR+CTC analysis; (3) the interval between sampling and surgery was more than 7 days; and (4) prior cancer history. All the procedures were conducted in accordance with the Helsinki Declaration and the study was approved by the institutional ethics committee of the Shanghai Pulmonary

Hospital. Written informed consent was obtained from all participants upon enrollment.

2.2 | Data collection

Data were collected from the clinician records through the standard case report form (CRF), including demographic characteristics, clinical symptoms, surgical methods, and CT image information.

2.3 | CT scan

All participants received a chest CT scan (Revolution CT, GE Healthcare,) before inclusion into this study. CT image characteristics including lesion size, type, and location were observed and agreed upon by at least two experienced radiologists. A multidisciplinary team then evaluated the malignancy of the identified nodules. Patients with nodules likely to be malignant subsequently undergo surgical resection.

2.4 | Pathological assessment

Resected tumors specimens were pathologically assessed by at least two experienced pathologists to determine the tumor invasiveness according to the IASLC/ATS/ERS classification of lung adenocarcinoma.² In brief, AAH was defined as a localized proliferation of less than or equal to 5 mm; AIS was defined as a localized lesion of less than or equal to 30mm with a pure lepidic pattern; MIA was defined as a solitary adenocarcinoma of less than or equal to 30mm with a predominantly lepidic pattern and less than or equal to 5mm invasion; and IA was defined as an adenocarcinoma lesion with other/mixed subtype and invasion.

2.5 | FR + CTC enumeration

Upon enrollment, 3 ml of peripheral venous blood was collected from each participant. FR+CTC level was analyzed by using the Cytoplorare® kit (Genosaber Biotech,) according to the manufacturer's protocol. Detailed procedures were as previously described.¹⁵ Briefly, the first step negatively enriches CTCs through lysis of red blood cells and depletion of white blood cells using antibody-coated magnetic beads. The second step labels FR+CTCs with a folate receptor alpha-targeting detection probe containing a specific oligonucleotide sequence and detects its signal using quantitative polymerase chain reaction (qPCR). A serial of diluted oligonucleotide samples corresponding to $2-2 \times 10^5$ FR+CTCs served as standards to calibrate the FR+CTC level. The unit used for FR+CTC quantitation was defined as folate receptor unit (FU)/3 ml of peripheral blood.

2.6 | Statistical analysis

Continuous variables were compared using Mann-Whitney *t*-test. Categorical outcomes were compared using Fisher's exact test and chi-square test. Receiver operating characteristic (ROC) curve was used to analyze the diagnostic performance of FR+CTC. Youden index was calculated to determine the optimal cutoff values. Multivariate analyses assessing predictors of precursor lesions (adjusted for gender, age, total number of pulmonary nodules, type of nodules, maximum tumor diameter [MTD], and location) was performed using logistic regression. Statistical analysis was performed by using Prism 8 (GraphPad Software,) and R 4.0.0 (R Foundation for Statistical Computing.).

3 | RESULTS

3.1 | Baseline characteristics

A total of 380 patients were enrolled in this study with indeterminate pulmonary nodules. 26 patients were excluded: 2 did not undergo surgery for physical reasons, 15 for blood sample reason (5 due to lack of operation time information, it is impossible to determine whether the blood was collected before surgery, 3 due to postoperative blood, 3 due to the interval between sampling and surgery was more than 7 days and 4 blood clots), 8 had uncertain pathological diagnosis, and 1 had prior cancer history. 354 patients underwent surgery and CTC analysis and were included in the final analysis (Figure S1). Of these 354 patients, 57 had benign lung disease, 133 were confirmed to precursor lesions, 136 were adenocarcinoma, and the remaining 28 had unspecified pathological types. Patient demographics and detailed clinical characteristics are shown in Table 1.

3.2 | FR + CTC level in determining precursor lesions

The median FR+CTC level of the precursor lesions group was higher than that of the benign group (10.60 vs. 7.52 FU/3 ml) (Table S1), the difference was statistically significant ($p < 0.001$) (Figure 1A). FR+CTC can effectively differentiate precursor from benign lesions (cutoff value: 9.22 FU/3 ml; sensitivity: 69.17%; specificity: 82.46%) (Figure 2A, Table 2). The median FR+CTC levels of the precursor lesions and the malignant lesions group were 10.60 FU/3 ml and 10.44 FU/3 ml, respectively (Table S1). The difference was not statistically significant ($p = 0.71$) (Figure 1A), FR+CTC cannot differentiate precursor lesions from malignant tumors (cutoff value: 11.61 FU/3 ml; sensitivity: 63.97%; specificity: 44.36%) (Figure 2B, Table 2). The median FR+CTC levels of the single pulmonary lesion and the multiple pulmonary lesions group were 10.15 FU/3 ml and 9.33 FU/3 ml, respectively (Figure 1B). And the median FR+CTC levels of the benign, the precursor lesion, and malignant tumors group in single

TABLE 1 Patients' demography of 354 eligible patients

Number of patients	N (%)
Gender	
Male	135 (38.1)
Female	219 (61.9)
Age (SD), years	50.7 (11.5)
Total number of pulmonary nodules identified ^a	
1	277 (78.2)
>1	76 (21.5)
Pathological subtype	
Benign	57 (16.1)
Precursor lesions	133 (37.6)
Malignant lesions	136 (38.4)
Complex pathology ^b	28 (7.9)
Type of nodules	
Pure GGN	276 (78.0)
Pure-solid GGN	20 (5.6)
Solid nodule	36 (10.2)
Location	
Upper	180 (50.9)
Middle	32 (9.0)
Lower	107 (30.2)
Surgery	
Wedge resection	123 (34.7)
Segmentectomy	129 (36.4)
Lobectomy	64 (18.1)
Complex ^c	38 (10.7)

^aone case no data.

^bboth precursor and malignant lesions are present in multiple lesions cases.

^ccontains at least two of the above three resections.

pulmonary lesion patients were 7.37 FU/3 ml, 10.60 FU/3 ml, and 10.55 FU/3 ml (Table S1, Figure 1C). The difference between benign and precursor was statistically significant ($p = 0.004$), while the difference between precursor and malignant was not statistically significant ($p = 0.57$). FR+CTC can effectively differentiate precursor lesions from benign lesions (cutoff value: 9.03 FU/3 ml; sensitivity: 75.20%; specificity: 83.00%) (Figure 2C, Table 2) and FR+CTC cannot differentiate malignant tumors from precursor lesions (cutoff value: 13.13 FU/3 ml; sensitivity: 76.32%; specificity: 31.86%) (Figure 2D, Table 2). The median FR+CTC levels of the benign, the precursor glandular lesion, and malignant tumors group in multiple pulmonary lesion patients were 7.88 FU/3 ml, 10.44 FU/3 ml, and 9.39 FU/3 ml (Figure 1D). For patients with multiple pulmonary lesions, FR+CTC neither differentiate precursor lesions from benign lesions (cutoff value: 12.64 FU/3 ml; sensitivity: 88.89%; specificity: 45.00%) (Figure 2E, Table 2) nor precursor lesions from malignant tumors (cutoff value: 12.38 FU/3 ml; sensitivity: 72.73%; specificity: 45.00%) (Figure 2F, Table 2).

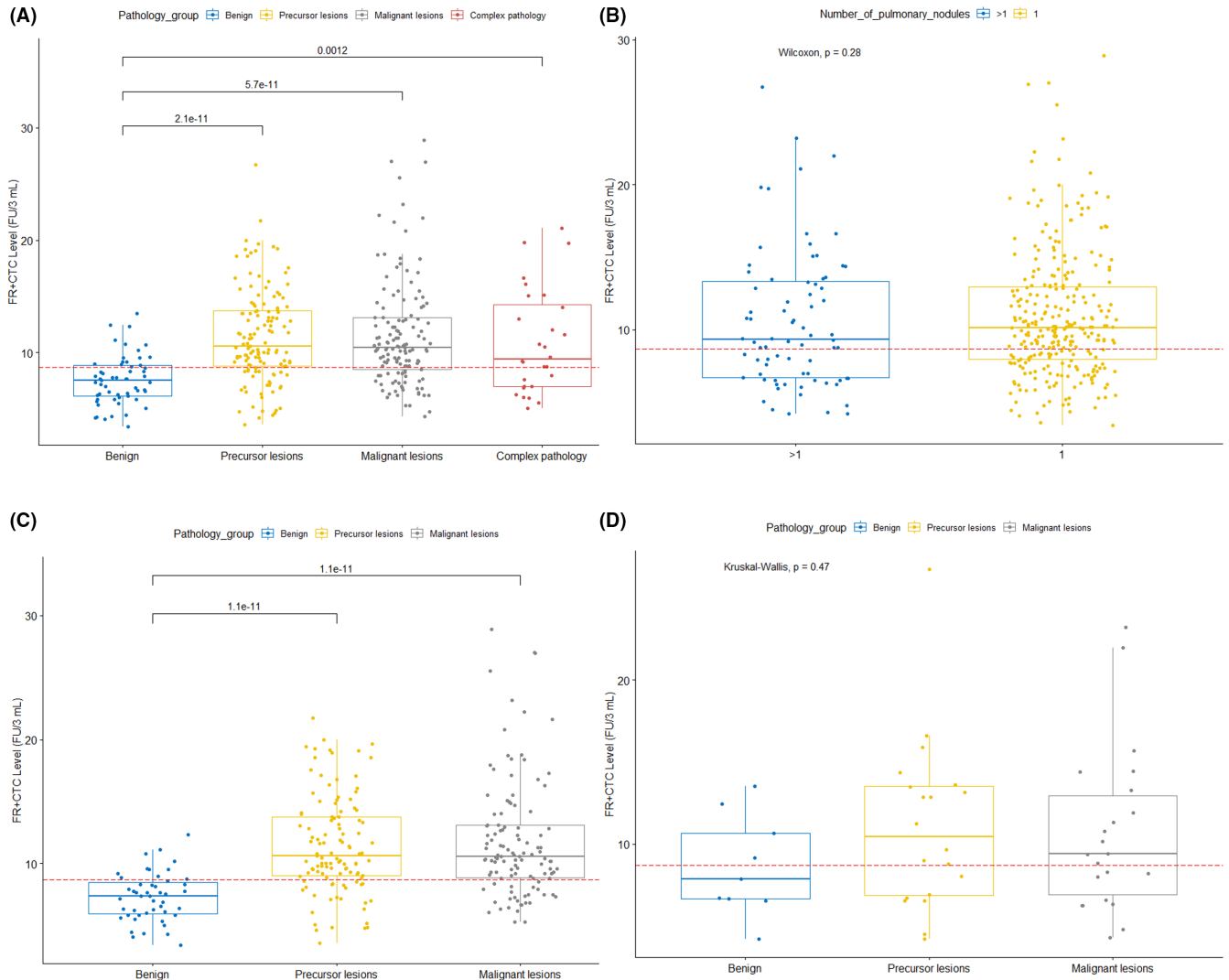


FIGURE 1 FR+CTC levels in precursor glandular lesion and different pathological subtypes of lung adenocarcinoma. (A) Comparison of the FR+CTC levels between patients with benign/precursor lesions/malignant lesions and complex pathology; (B) Comparison of the FR+CTC levels between patients with single and multiple pulmonary lesions; (C) Comparison of the FR+CTC levels between benign/precursor lesions/malignant lesions in patients with single pulmonary lesion; (D) Comparison of the FR+CTC levels between benign/precursor lesions/malignant lesions in patients with multiple pulmonary lesions

3.3 | Univariate and multivariate analysis for precursor lesions

To identify independent factors that influence the differentiation between malignant lesions and precursor lesions, the univariate analysis indicated that age, total number of pulmonary nodules, MTD, location (lower vs upper) were statistically significant (all $p < 0.05$), and for multivariate analysis, multiple pulmonary lesion was 3.104 times more likely to malignant lesions than the single multiple pulmonary lesions (AOR: 3.104, 95% CI: 1.525, 6.316), the MTD ≥ 1 cm was more likely to be malignant lesions (AOR: 3.148, 95% CI: 1.722, 5.754), lower lobe were more likely to malignant lesions than upper lobe (AOR: 2.098, 95% CI: 1.132, 3.888) (Table 3).

4 | DISCUSSION

Based on accurate preoperative diagnosis, physicians can help patients in deciding whether the patient should undergo surgical treatment and the extent of the surgery. However, there is a lack of effective methods to differentiate precursor lesions from benign pulmonary diseases or other pathological subtypes of lung cancer currently. Due to their good prognosis, AIS are considered low-grade malignant tumors.²⁰ However, since most patients with lung cancer exhibit middle or late stage disease at the initial visit, early detection, early diagnosis, and correct staging play an extremely important role in improving their survival rate. Therefore, noninvasive, preoperative differentiation of precursor lesions from benign or malignant is essential in guiding the clinical management of these patients.²¹

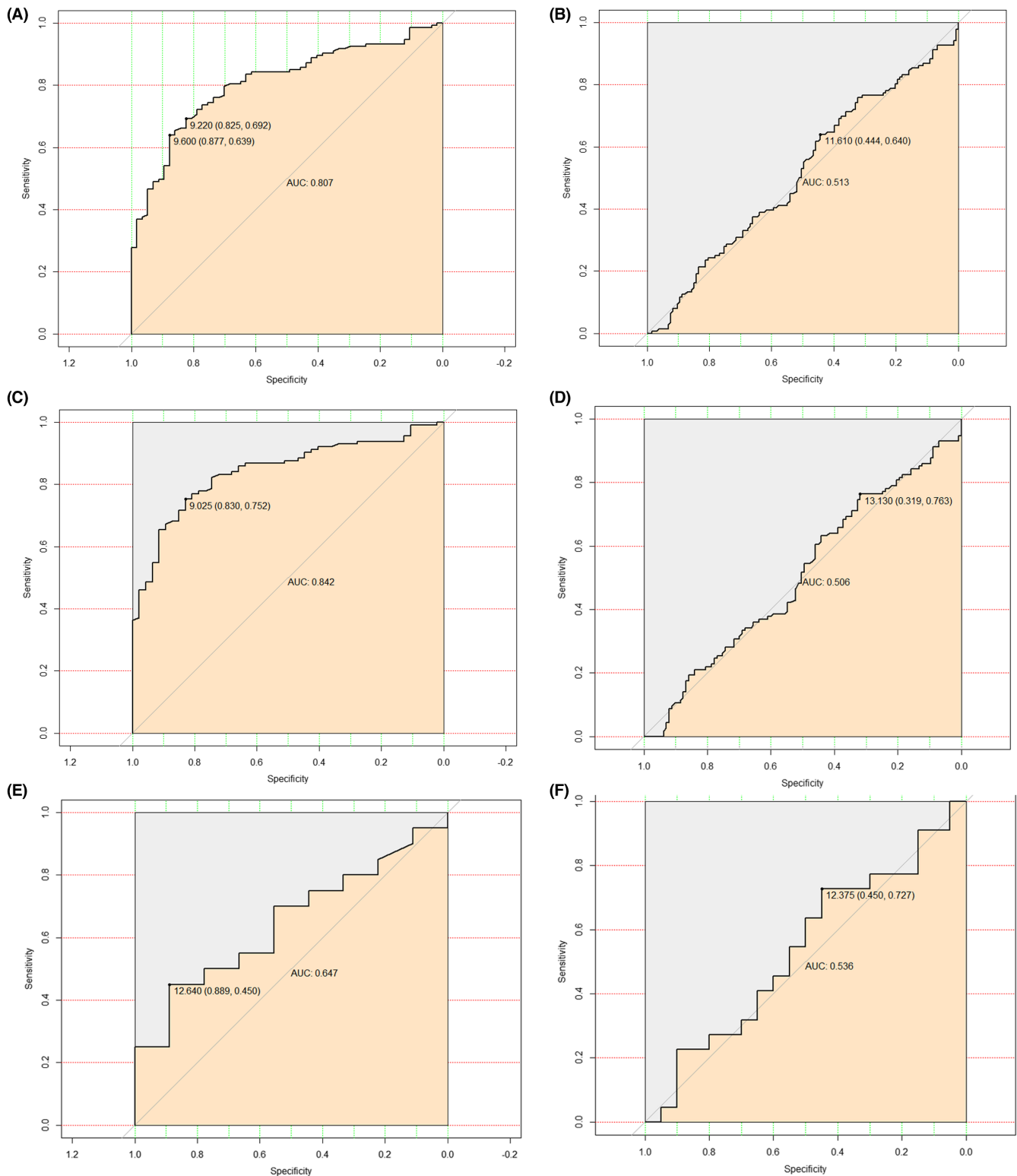


FIGURE 2 ROC curves. (A) The performance of FR+CTC in differentiating precursor glandular lesions from benign; (B) The performance of FR+CTC in differentiating precursor glandular lesions from malignant; (C) The performance of FR+CTC in differentiating precursor glandular lesions from benign in single pulmonary lesions; (D) The performance of FR+CTC in differentiating precursor glandular lesions from malignant in single pulmonary lesions; (E) The performance of FR+CTC in differentiating precursor glandular lesions from benign in multiple pulmonary lesions; (F) The performance of FR+CTC in differentiating precursor glandular lesions from malignant in multiple pulmonary lesions.

TABLE 2 Results of ROC analysis

	Area under curve	95% confidence interval	p value	Cutoff	Sensitivity	Specificity
Benign vs precursor	0.8069	0.7441–0.8697	<0.0001	9.220	0.6917	0.8246
Precursor vs malignant	0.5130	0.4435–0.5824	0.7145	11.610	0.6397	0.4436
Benign vs precursor (single lesions)	0.8417	0.7800–0.9035	<0.0001	9.025	0.7520	0.8300
Precursor vs malignant (single lesions)	0.5065	0.4308–0.5822	0.8667	13.13	0.7632	0.3186
Benign vs precursor (multiple lesions)	0.6472	0.4385–0.8559	0.1101	12.64	0.8889	0.45
Precursor vs malignant (multiple lesions)	0.5364	0.3563–0.7164	0.6922	12.375	0.7273	0.45

TABLE 3 Univariate and multivariate analysis for precursor glandular lesions

Factors	Univariate analysis		Multivariate analysis	
	OR, 95% CI	p value	OR, 95% CI	p value
FR + CTC level (≥ 11.615)	0.69(0.43–1.1)	0.12	0.7276(0.416–1.273)	0.2653
Gender (Female)	0.82(0.5–1.33)	0.42	–	–
Age (≥ 60)	2.36(1.36–4.08)	<0.01	1.840(0.972–3.516)	0.0610
Total number of nodules(>1)	2.33(1.3–4.18)	<0.01	3.104(1.525–6.316)	0.0018
MTD (≥ 1 cm)	3.76(2.24–6.32)	<0.01	3.148(1.722–5.754)	<0.01
Solid nodule	Reference			
Pure solid GGN	3,139,272(0–Inf)	0.98	6,934,000(0–Inf)	0.9882
Pure GGN	0.23(0.03–1.98)	0.18	0.742(0.029–2.599)	0.2595
Location				
Upper	Reference			
Middle	0.77(0.34–1.75)	0.53	0.836(0.325–2.148)	0.710
Lower	1.82(1.05–3.17)	0.03	2.098(1.132–3.888)	0.0186

MTD: Maximum tumor diameter.

In the present study, median FR+CTC of the malignant group was 10.44 FU/3 ml, which was significantly higher than 7.52 FU/3 ml of the benign group. We confirm that whether the multiple nodules have the same infiltration property or not, in the multiple lesions subgroup, median FR+CTC of the malignant group was 9.39 FU/3 ml, while that of the benign group was 7.88 FU/3 ml, the result was similar. However, due to the sample size being too small and the lack of more genotypes and other data, it is impossible to determine whether they are multiple primary or intra pulmonary metastasis. The differences in their biological characteristics lead to complex effects on CTC level, but the overall judgment of benign and malignant by CTC is not affected (in single lesion group). The FR+CTC levels between benign and precursor lesions in patients with single pulmonary lesion is significantly different ($p < 0.001$); while FR+CTC levels between precursor lesions and malignant lesions is not statistically different ($p > 0.05$). In this group of patients, benign and precursor lesions can be distinguished, while precursor lesions and malignant lesions was not well differentiated, especially in the multiple lesions group, the possible reason is small sample size, and we need further studies. Recently, Hu et al. reported that early spread of colorectal cancer usually occurs at a very early stage when the basement membrane is not breached and the cancer is undetectable in clinical (usually less than 0.01cm^3), although the mechanism is still unclear, it is likely to be detected by CTC.²²

Since the report from the North American Lung Cancer Study Group of a three-fold increase in local recurrence rate and a decrease in overall survival following limited resection, lobectomy has been the standard of care for early-stage nonsmall cell lung cancer (NSCLC) in the past two decades,²³ but non-IA patients may be candidates for limited surgical resection.²¹ Nevertheless, recent studies have shown that sublobar resection could be acceptable for patients with noninvasive adenocarcinomas.²⁴ For patients with stage IA NSCLC, segmentectomy has showed a comparable survival and a better preservation of lung function.^{24,25} Indeed, the NCCN guideline suggests that sublobar resection is appropriate in patients with peripheral nodule ≤ 2 cm, and pure adenocarcinoma in situ (AIS) histology. Therefore, development of a noninvasive and sensitive strategy for predicting the invasiveness of lung nodules, particularly in NSCLC, before selecting eligible patients for limited lung resection will significantly benefit patients with noninvasive lung carcinoma, particularly early-stage, undefined small-sized SPNs, by avoiding major lung resections. In the long run, a reliable differentiating strategy for SPNs will significantly contribute to the reduction of lung cancer-associated mortality by guiding adequate and timely treatment and avoiding overtreatment of noninvasive SPNs. Our result of FR+CTC level cannot in favor of distinguishing precursor lesions and malignant lesions, and because of that we explored the risk factors

of malignant, the results showed that MTD >10mm were independent risk factors for malignant lesions, it is consistent with previous study, however, location (lower lobe) is inconsistent. Total number of nodules > 1 was showed to be an independent risk factor for malignant lesions, and the age was almost an independent risk factor in our study ($p = 0.061$). Zhou et al. demonstrated that in small-sized lung adenocarcinoma, FR+CTC level was significantly lower in non-invasive adenocarcinomas than that in invasive adenocarcinomas. And multivariate analysis revealed that smaller tumor size and lower FR+CTC level are significant independent differentiators of noninvasive cancer from invasive cancer.¹⁸ With the excessive care in lung cancer screening, an efficacious biomarker for assisting LDCT to determine the malignancy and tumor invasiveness of lung nodules is urgently warranted.²⁶ Increasing effort is being made to leverage FR α over expression as a diagnostic marker and/or to enable more precise cancer surgery. Although lobectomy is the standard surgical procedure for lung cancer, a limited resection is suggested for some carefully selected patients, in particular, patients with small-sized lesions. A previous report suggested that noninvasive adenocarcinomas are potential candidates for limited resection and have excellent prognosis.²⁷ In practice, it is sometimes difficult to eliminate tumor invasion through intraoperative morphological diagnosis based on frozen sections.²⁸ Lee et al. reported that lesion border and lesion margin on CT imaging that were routinely used in clinical settings showed only moderate interobserver agreement,²⁹ thereby calling for a more objective and robust parameter other than CT imaging to differentiate noninvasive adenocarcinomas from invasive ones. Preoperative CTC detection combined with the maximum tumor diameter can effectively evaluate the subnodular infiltrates and the subnodular tumoticlea like IPA.¹⁸

Several limitations of the current study should be mentioned, firstly, the present study was a retrospective study although the CTC was collected prospectively, it resulted in the loss of crucial information and information bias, especially imaging morphological information, furthermore, we were unable to screen predictors based on morphology and adjust for some potential confounders. Secondly, the small number of included patients, and the difference in the number between the benign and precursor or malignant groups, increase uncertainty.

5 | CONCLUSIONS

FR + CTC can be identified in precursor glandular lesions and utilized to differentiate from benign pulmonary diseases. Total number of pulmonary nodules, MTD, location (lower vs upper) were independent risk factors for malignancy. FR + CTC is expected to be an alternative biomarker for preoperative surgical decision making. However, further studies with large samples are needed for confirmation.

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CONFLICT OF INTEREST

The author has no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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