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ORIGINAL ARTICLE

FGB and FGG derived from plasma exosomes as potential biomarkers to distinguish benign from malignant pulmonary nodules

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Abstract

Previous proteomic analysis (label-free) of plasma exosomes revealed that the expression of FGG and FGB was significantly higher in the malignant pulmonary nodules group, compared to the benign pulmonary nodules group. The present study was performed to evaluate the role of plasma exosomal proteins FGB and FGG in the diagnosis of benign and malignant pulmonary nodules. We examined the expression levels of FGB and FGG in plasma exosomes from 63 patients before surgery. Postoperative pathological diagnosis confirmed that 43 cases were malignant and 20 cases were benign. The ROC curve was used to describe the sensitivity, specificity, area under the curve (AUC) of the biomarker and the corresponding 95% confidence interval. We confirmed that the expression levels of FGB and FGG were higher in the plasma exosomes of malignant group than in the benign group. The sensitivity and AUC of FGB combined with FGG detection to determine the nature of pulmonary nodules are superior to single FGB or FGG detection. FGB and FGG might represent novel and sensitive biomarker to distinguish benign from malignant pulmonary nodules.

Keywords Exosome · Pulmonary nodules · FGB · FGG

Abbreviations

PNs	Pulmonary nodules
FGB	Fibrinogen beta chain
FGG	Fibrinogen gamma chain
ROC	Receiver operating characteristic
AUC	Area under curve
CT	Computed tomography
PET	Positron emission computed tomography

Introduction

Lung cancer is one of the most common malignant tumors in the world, and the incidence rate and tumor mortality rate are among the highest in malignant tumors [1]. A considerable part of lung cancer patients have early imaging findings of pulmonary nodules, so differential diagnosis of benign and malignant pulmonary nodules is important for the development of follow-up treatment options [2]. In addition to clinical experience, clinicians use the following methods to evaluate and manage the benign and malignant pulmonary nodules: the American College of Chest Physicians (ACCP) guideline [3], VA model [4], Svensson Model [5] and so on. However, for the above methods, it is difficult to achieve satisfactory results in the clinical practice of identifying benign and malignant pulmonary nodules. Clinical low-dose CT has limited ability to judge the benign and malignant pulmonary nodules. The main method for preoperative diagnosis of benign and malignant pulmonary nodules is lung tissue biopsy, but this invasive examination not only causes damage to the patient's body, but also increases the risk of tumor metastasis, and there is a certain false negative rate [6]. Based on the limitations of these detection methods, it is urgent to find a method that is accurate and has little damage to the body to identify the benign and malignant pulmonary nodules.

In recent years, the rapid development of omics technology and liquid biopsy technology has provided a new perspective for diagnosing diseases [7–10]. The emergence of

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liquid biopsy makes up for the lack of tissue biopsy, opening up a new era of cancer detection, and becoming one of the diagnostic techniques representative of precision medicine [11]. In addition to being noninvasive, liquid biopsy has the advantages of being fast and simple while avoiding the lack of tissue biopsy samples. In addition, researchers can continuously monitor disease through liquid biopsy [12]. For patients, liquid biopsies also protect them from radiation. For example, CT or PET scans can cause radiation [13], and liquid biopsies allow patients to avoid such radiation, allowing researchers to monitor and identify tumors with minimal damage. Liquid biopsy includes detection and analysis of circulating tumor cells [14], circulating DNA [15] or exosomes [16], etc., which can reflect the characteristic changes in lesions in patients in real time and have good reproducibility. Exosomes can carry a large amount of nucleic acid, lipids and proteins of their host cells, and are freed in the circulatory system [17]. Our previous study screened a series of proteins with significant differences by collecting exosomes in the plasma of patients with benign and malignant pulmonary nodules and performing protein profiling [18]. Compared with the benign nodule group, Fibrinogen beta chain (FGB) and fibrinogen gamma chain (FGG) were highly expressed in the plasma exosomes of patients with malignant pulmonary nodules (differential times > 1.5 times, $p < 0.05$). Our current study further validates these two differential proteins and finds that these two proteins can better distinguish between benign and malignant pulmonary nodules. As far as we know, the role of these two proteins in the diagnosis of pulmonary nodules has not been reported. Our findings provide new biomarkers for the diagnosis of benign and malignant pulmonary nodules.

Methods

Patients and samples

A total of 63 patients were enrolled in present study, including 20 patients with benign pulmonary nodules and 43 patients with malignant pulmonary nodules. These patients were treated with surgery in our institution. The final diagnosis is based on the pathological diagnosis of the surgically removed sample. The patient's whole blood sample was collected before surgery. All patients signed the informed consent. The collection of samples was reviewed by the hospital ethics committee.

Exosomes extraction and identification

Eight millimeters whole blood sample of each PN patient was collected using a test tube containing EDTA before surgery. The plasma was centrifuged within 1 h of blood collection, centrifuged at 3000 *g*, 4 °C, 5 min, and then the supernatant was taken for extraction of exosomes. Plasma exosomes were isolated according to the exoEasy Maxi Kit (Qiagen) kit instructions. Electron microscopy and western detection were used to identify exosomes.

Western blot

Exosomes were lysed using sample buffer, and proteins were quantified using BCA reagent for exosomal proteins. Thirty micrograms of protein was used for SDS-PAGE separation, then transferred to a PVDF membrane, and the membrane was blocked for 1 h using TBST to prepare a 5% concentration of fat-free milk. The primary antibody (CD63:ab59479 Abcam, FGB: ab232793 Abcam, FGG: ab119948 Abcam) was incubated overnight, and after incubation with the secondary antibody, it was developed using a chemoluminescent agent (Thermo Pierce ECL Western Blotting Substrate).

Statistical analysis

Quantity one was used to evaluate the results of western blot, and the relative expression of the protein was the abundance of the protein compared to the abundance of its internal reference protein. The ROC curve was used to describe the sensitivity, specificity, area under the curve (AUC) of the biomarker and the corresponding 95% confidence interval. The *t* test was used to analyze the relationship between the clinicopathological features of patients and the benign and malignant pulmonary nodules, and p value < 0.05 was considered to be significant. Binary logistic regression was used for the joint analysis of the two markers. The software used in this study is Graphpad Prism 5 and SPSS 20.

Results

Research cohort

In present study, we collected blood samples from patients with pulmonary nodules. Representative CT images and pathological images of benign and malignant pulmonary nodule patients are shown in Fig. 1a and b, respectively. In total, we included 106 patients with pulmonary nodules, in previous study, 40 patients were included in discovery group, and 63 patients were enrolled in this validation group (Fig. 2a).

In the current study cohort, there was no significant difference in gender, age and smoking status between benign and malignant pulmonary nodule patients (Table 1). However, it can be found that in both benign and malignant pulmonary nodules, the proportion of non-smokers is higher than that of smokers.

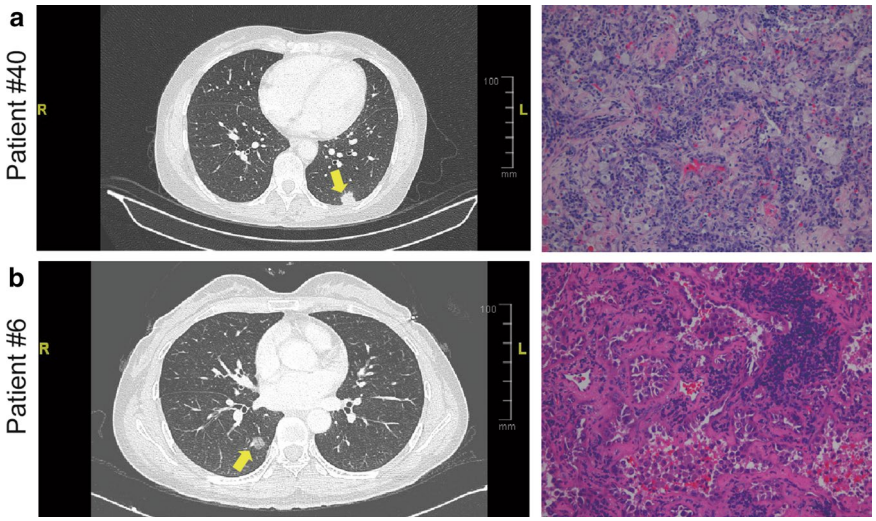

Fig. 1 Representative CT images of patients with pulmonary nodules and HE staining of pathological sections. **a** Representative CT image of a patient (#40) with benign PN who was diagnosed with interstitial pulmonary fibrosis with lymphocytes infiltration. Corresponding paraffin-embedded tissue was processed for H&E staining (200×). **b** Representative CT image of a representative patient (#6) with malignant PN who was diagnosed with lung adenocarcinoma (lepidic predominant); corresponding paraffin-embedded tissue was processed for H&E staining (200×)

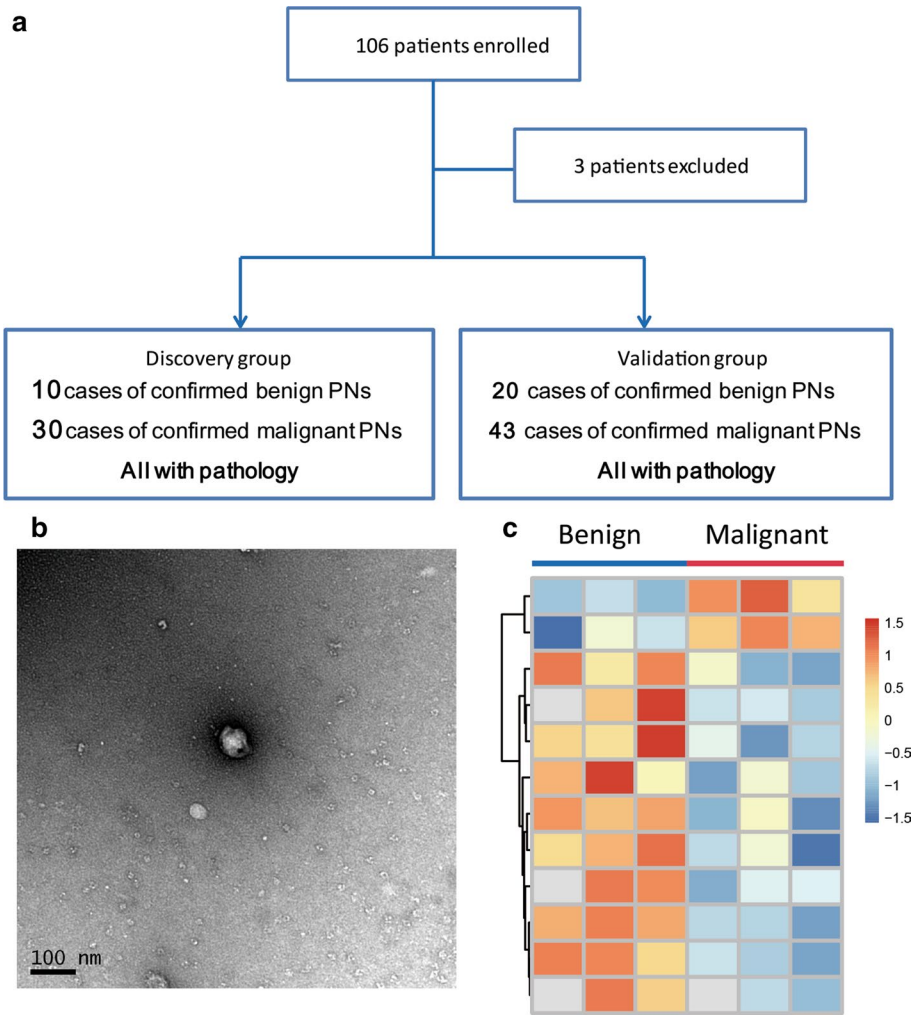

Fig. 2 Study design and screening for differentially expressed proteins in plasma exosomes in patients with benign and malignant pulmonary nodules. **a** Study design. **b** Exosomes isolated from plasma were characterized by electron microscopy. **c** Heatmap of differentially expressed proteins in plasma exosomes in patients with benign and malignant pulmonary nodules, fold change > 1.5 times

Table 1 Clinicopathologic features of patients in validation group

Features	Benign PNs ($n=20$)	Malignant PNs ($n=43$)	p value
Sex			0.952
Male	9 (32.1%)	19 (67.9%)	
Female	11 (31.4%)	24 (68.6%)	
Age			0.777
≤Median	11 (33.3%)	22 (66.7%)	
>Median	9 (30.0%)	21 (70.0%)	
Smoking			0.896
Yes	4 (33.3%)	8 (66.7%)	
No	16 (31.4%)	35 (68.6%)	
Histologic diagnosis			–
Lung adenocarcinoma		43 (100%)	
Granuloma	4 (20%)		
Inflammation	5 (25%)		
Hamartoma	3 (15%)		
Dysplasia	4 (20%)		
Other	4 (20%)		

Median age: 60.5 years old

Characteristics of exosomes

We observed the extracted plasma-derived exosomes by transmission electron microscopy. The extracted exosomes were round in shape, and the exosomal vesicles were approximately 100 nm in diameter (Fig. 2b). In addition, we performed a western blot analysis of the exogenous marker protein CD63. The results showed that the extracted vesicles possessed the characteristics of exosomes.

Comparison of the proteomes of malignant/benign PNs plasma exosomes

We have previously detected a series of proteins differentially expressed in benign and malignant pulmonary nodule tissues by mass spectrometry [18]. We found that there were significant differences in the expression levels of 12 proteins in the benign and malignant PNs groups (multiplying multiples of 1.5 times and $p<0.05$) (Fig. 2c). In the current study, we selected two proteins, FGB and FGG, which were relatively highly expressed in malignant nodules, for further validation analysis (difference multiples > 1.5, $p<0.05$). Gene cooccurrence of FGB and FGG was analyzed by STRING [19] (Fig. 3a). We performed a western blot test for FGB, FGG and CD63 in plasma exosomes of patients with pulmonary nodules (Fig. 3b). By analyzing the grayscale of the bands, we found that the expression levels of FGB and FGG were higher in the plasma exosomes of patients with malignant pulmonary nodules than in the benign nodules group (Fig. 3c and d).

Exosomal proteins FGB and FGG in pulmonary nodule prognosis

To validate the clinical identification of these two biomarkers, we used ROC curves for independent and pooled analysis of these two proteins. The ROC curve was used to assess the ability of FGB, FGG and FGB combined with FGG to diagnose benign and malignant pulmonary nodules (Fig. 4a and b). As shown in Table 2, the diagnostic sensitivity of the FGB in the current cohort was 0.628, the specificity was 0.800, and the AUC was 0.741 (95% CI 0.616–0.866, $p=0.002$). The diagnostic sensitivity of FGG in the current cohort was 0.535, the specificity was 0.850, and the AUC was 0.659 (95% CI 0.526–0.793, $p=0.043$). Then we performed logistic regression analysis on the two proteins, and obtained the combined sensitivity of the two proteins as 0.814, specificity as 0.700, and AUC as 0.794 (95% CI 0.681–0.908, $p<0.001$).

Discussion

More than one million people worldwide are diagnosed with pulmonary nodules each year, and a significant proportion of them are malignant pulmonary nodules [20]. For early intervention in malignant pulmonary nodules and for avoiding excessive treatment of benign pulmonary nodules, early identification of the nature of pulmonary nodules is particularly important for the choice of treatment options. Pulmonary nodule tissue biopsy detection methods have invasive, false negative and may cause tumor dissemination and other shortcomings, and we need a safer, simpler and more reliable detection method [21]. In recent years, the development of testing techniques has provided many useful biomarkers for the diagnosis of patients with pulmonary nodules. Studies have shown that the expression of VEGF-C in plasma combined with CT or PET has a certain value in the diagnosis of pulmonary nodules [22]. There were also reports that the combination of multiple biomarkers can help identify the benign and malignant pulmonary nodules. Neuron-specific enolase (NSE) [23], cytokeratin fragment antigen 21-1 (CYFRA21-1) [24] and carcinoembryonic antigen (CEA) [25] and other serological markers have been used in clinical work, but the specificity was not high, and its role in the early identification of pulmonary nodules was controversial.

Exosome detection, as a component of liquid biopsy, provides new candidate biomarkers for clinical work to distinguish between benign and malignant pulmonary nodules [26]. Exosomes carry a large amount of protein information from host cells, including oncoproteins, receptors, kinases,

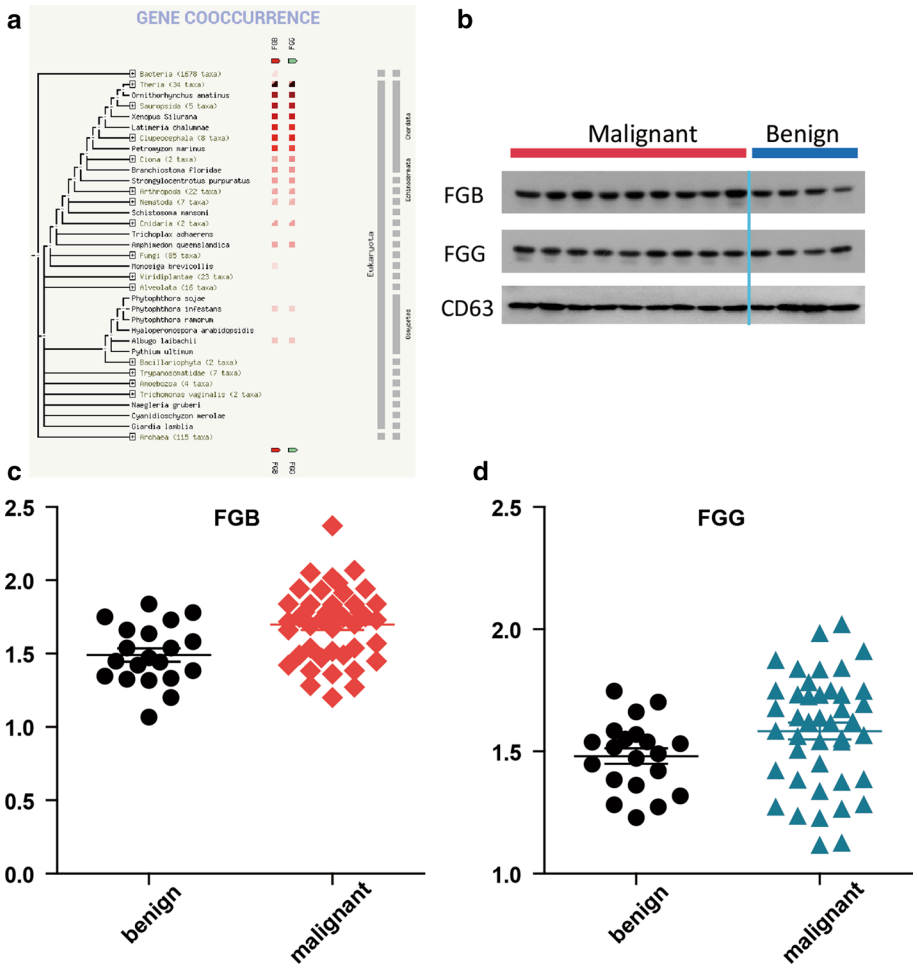

Fig. 3 Compared analysis of FGB and FGG protein expression in plasma exosomes of patients with benign and malignant nodules. **a** Gene cooccurrence of FGB and FGG, data from STRINGS (https://string-db.org/). **b** Representative pictures of western detection of FGB, FGG and CD63 in plasma exosomes of patients with pulmonary nodules. The left panel of the figure shows the expression of FGB, FGG and CD63 in the malignant pulmonary nodule group, and the right panel shows the expression of FGB, FGG and CD63 in the benign pulmonary nodule group. **c**, **d** Relative expression of FGB and FGG in plasma exosomes of patients with benign and malignant pulmonary nodules

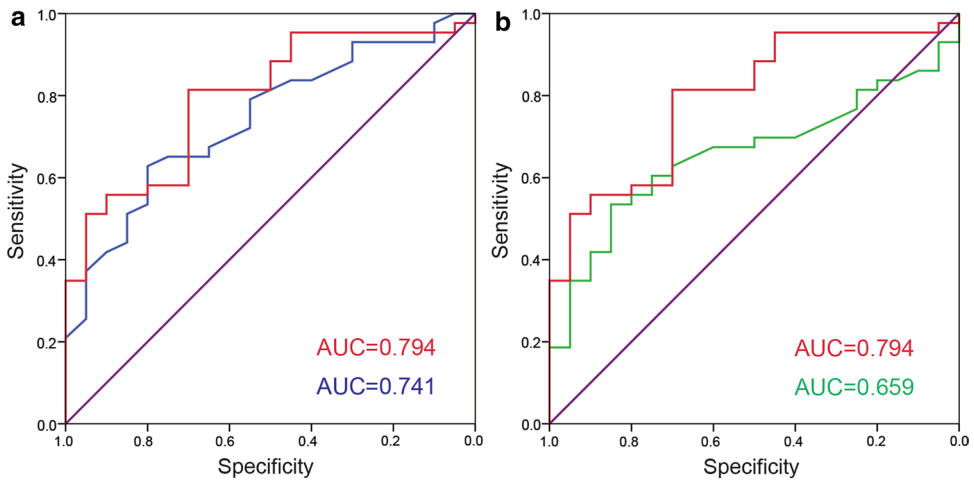

Fig. 4 ROC curves for FGB, FGG and FGB/FGG in the PN-diagnosis cohorts. **b** The blue line in the figure indicates the ROC curve of FGB for the prediction of benign and malignant lung nodules, AUC = 0.741. The red line indicates the ROC curve of FGB combined with FGG for the prediction of benign and malignant lung nodules, AUC = 0.794. **b** The green curve represents the ROC curve of FGG for the prediction of benign and malignant pulmonary nodules, AUC = 0.659. The red line indicates the ROC curve of FGB combined with FGG for the prediction of benign and malignant lung nodules, AUC = 0.794 (colour figure online)

Table 2 Efficacy of FGB, FGG and FGB + FGG in the diagnosis of lung nodules using ROC curve

Indicator	AUC (95% CI)	Sensitivity%	Specificity%	*p* value
FGB	0.741 (0.616–0.866)	62.8	80.0	0.002
FGG	0.659 (0.526–0.793)	53.5	85.0	0.043
FGB + FGG	0.794 (0.681–0.908)	81.4	70.0	< 0.001

cytokines, and the like [27]. Detecting the characteristics of proteins in exosomes can better reflect the overall development of tumorigenesis compared to the detection of proteins in plasma. For example, EGFR has been reported to be detected in plasma exosomes in the early stages of lung cancer [28]. At present, most studies were aimed at the early diagnosis of lung cancer by exosome protein, and there were few studies on the detection of benign and malignant pulmonary nodules by exosome protein detection. The clinical application of exosome proteins FGG and FGB in the differential diagnosis of benign and malignant pulmonary nodules has not been reported. In present study, we included 63 patients who were difficult to identify the benign and malignant nodules by imaging. We performed a clinical validation analysis of differentially expressed proteins FGB and FGG in plasma exosomes of benign and malignant nodules found in previous studies. The results showed that the combined analysis of FGB and FGG had clinical value in the differential diagnosis of benign and malignant pulmonary nodules (sensitivity = 0.814, specificity = 0.700, AUC = 0.794, 95% CI 0.681–0.908). Our findings provided new markers for the differential diagnosis of benign and malignant pulmonary nodules.

FGB, FGG and FGA can be polymerized to form an insoluble fibrin matrix fibrinogen (FBG). As one of the main components of blood clots, the main function of fibrin is coagulation [29]. In addition, fibrin deposition is also associated with infection [30] and can also promote antibacterial immune responses [31] through both innate and T cell-mediated pathways. Both FGB and FGG were target genes for IL-6 and STAT3 signaling pathways [32]. In recent years, the role of FGB and FGG in tumors has gradually been revealed. Endogenously synthesized fibrinogen can promote the growth of lung and prostate cancer cells [33]. In lung cancer, FGB and FGG were one of the key epithelial–mesenchymal transition effectors associated with cell adhesion and cellular communication [34]. FGB can be used as a biomarker for the diagnosis of bladder cancer [35]. In addition, FGB was also detected to be highly expressed in laryngeal cancer [36]. A combination of five urinary biomarkers (including FGB) can discriminate lung cancer patients from control groups, as well as other common tumors such as bladder cancer, colorectal cancer and so on [37]. Studies have used the TCGA database to screen a series of genes associated with the prognosis of patients with lung adenocarcinoma, of which FGB has been shown to be a prognostic marker for lung adenocarcinoma [38]. Compared with normal liver tissue, FGG is highly expressed in hepatocellular carcinoma, which can predict the clinical progression of hepatocellular carcinoma [39]. FGG in urine can also be used as a biomarker for prostate cancer [40]. Using the PRECOG website, we analyzed the expression levels of FGB and FGG in lung adenocarcinoma. The Z scores of FGB and FGG were found to be 2.82 and 2.24, respectively (https://precog.stanford.edu/index.php), indicating that the expression levels of FGB and FGG in lung adenocarcinoma tissues were higher than those in normal lung tissues. The ROC curve showed that the sensitivity of the pulmonary nodules was not ideal when using FGB or FGG alone to predict the benign and malignant pulmonary nodules. After the combined application of FGB and FGG, the sensitivity, specificity and AUC of the diagnosis of benign and malignant pulmonary nodules were improved compared with the application of a single protein. Our findings indicate that FGB and FGG in plasma exosomes as a combined marker can better predict the benign and malignant pulmonary nodules compared to FGB or FGG alone.

Conclusion

In summary, we reported for the first time the role of FGB and FGG from plasma exosomes in predicting benign or malignant pulmonary nodules. We found that the combination of FGB and FGG can efficaciously distinguish the benign and malignant pulmonary nodules with high sensitivity and specificity. Our findings provided a novel noninvasive method of identifying benign and malignant pulmonary nodules. Due to the limited sample size of the current study cohort, there may be bias in sample selection. We wish we could further verify the efficacy of combined detection of FGB and FGG in the differential diagnosis of benign and malignant pulmonary nodules in multiple centers in the future.

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Authors' contributions MK performed data analysis and wrote the manuscript; YP and XT carried out extraction and identification of exosomes. ZZ helped to perform bioinformatics analysis. LZ and HM collected samples and information of clinical cases. YS and HZ conceived of the study and participated in its designation and helped to draft the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval All procedures performed in the study involving human participants were in accordance with the ethical standards of the Committee for Ethical Review of Research of Fudan University and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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