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ORIGINAL ARTICLE

The Serum Tumor Markers in Combination for Clinical Diagnosis of Lung Cancer

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SUMMARY

Background: **To explore the clinical value of combined detection of serum tumor markers in lung cancer, including carcinoembryonic antigen (CEA), carbohydrate antigen 15-3 (CA15-3), cytokeratin 19 fragment (CYFRA 21-1), neuron specific enolase (NSE), and squamous carcinoma antigen (SCCA).**

Methods: **The expression levels were compared among groups, and the combined effects of these tumor markers in the diagnosis of lung cancer were analyzed. In addition, *EGFR* gene mutations were detected in some patients with NSCLC.**

Results: **There were 776 patients (age 59.78 ± 10.39 years) with lung cancer and 794 controls (age 58.26 ± 15.73 years) included in our study. In this study, tumor markers were detected in lung cancer patients and controls. Individual sensitivity of the tumor markers sorted in descending order were CEA > CYFRA21-1 > CA15-3 > NSE, and the specificities were NSE > CYFRA21-1 > CEA > CA15-3. The combination of CEA + CA15-3 + CYFRA21-1 + NSE ranked the highest in the sensitivity index (75.00%) and specificity index (98.61%) in lung cancer. In adenocarcinoma, the area under the ROC curve (AUROC) of CEA (0.665) and CYFRA21-1 (0.631) were higher than those of CA15-3 (0.559) and NSE (0.507). In squamous carcinoma, the AUC of CYFRA21-1 (0.722) and SCC (0.628) were higher than those of CEA (0.579), CA15-3 (0.524), and NSE (0.552). In small cell carcinoma, the AUC of NSE (0.654) was higher than those of CEA (0.616), CYFRA21-1 (0.555), and CA15-3 (0.482).**

Conclusions: **These serum tumor markers are valuable indicators in the clinical use. The combination of tumor markers can be used as a method to improve the effectiveness of clinical diagnosis for lung cancer.**

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KEY WORDS

lung cancer, non-small cell lung cancer, tumor markers

LIST OF ABBREVIATIONS

CEA - carcinoembryonic antigen
CA15-3 - carbohydrate antigen 15-3
CYFRA21-1 - cytokeratin fragment
NSE - neuron specific enolase
SCCA - squamous carcinoma antigen
EGFR - epidermal growth factor receptor
NSCLC - non-small cell lung cancer
IQR - interquartile range
ADK - adenocarcinomas
TNM - TNM classification of malignant tumors
AUC - area under the ROC curve

INTRODUCTION

Lung cancer has the highest morbidity and mortality in the world, and the prevalence of lung cancer is gradually rising [1-3]. Lung cancer is one of the most common causes of cancer death in men as well as women in some parts of the world (including North America, Eastern Asia, Northern Europe, Australia, and New Zealand) [4-8]. This may be related to tobacco smoking and environmental pollution. In some parts of the world, the tobacco epidemic continues to grow, contributing to an increase in the number of new lung cancer cases and deaths [9-11].

In February 2018, the China National Cancer Center released the latest issue of cancer statistics in China. The morbidity and mortality of male lung cancer ranks first in China. The age-standardized morbidity and mortality of male lung cancer in central China are the highest, which may be related to the higher smoking rate of males in China. The morbidity of lung cancer among women in China ranks second and the mortality rate of lung cancer among women is the first. This may be related to female exposure to environmental tobacco smoke, indoor oil fume and fuel pollution, and outdoor air pollution [11]. Lung cancer mortality remains serious and is likely to continue to rise in China. It also showed that the lung cancer mortality rates significantly increased with age (20 - 84) [12].

Early studies have shown that serum tumor markers have great significance in the detection, prognosis, and follow-up of lung cancer. These serum tumor markers such as carcinoembryonic antigen (CEA) [13], carbohydrate antigen 15-3 (CA15-3) [14,15], cytokeratin fragment (CYFRA21-1) [16,17], neuron specific enolase (NSE) [18,19] and squamous carcinoma antigen (SCCA) [20,21] have been studied in lung cancer patients. Somatic mutations in the activation of the epidermal growth factor receptor (EGFR) tyrosine kinase domain have been identified in a group of patients with advanced non-small cell lung cancer (NSCLC). Patients harboring these mutations in NSCLC show excellent response to EGFR tyrosine kinase inhibitors (EGFR-TKIs). The EGFR-TKI has been approved for the treatment of locally advanced or metastatic NSCLC in adult patients with EGFR-activated mutations.

There has been no systematic study on lung cancer patients and tumor markers in the Hakka population. In this study, the data of lung cancer patients who had detected tumor markers from 2017 to 2018, were analyzed retrospectively. In addition, the data of patients, in whom the *EGFR* gene had been detected, were analyzed, in order to explore the value of a combined application of serum tumor markers in clinical diagnosis for lung cancer.

MATERIALS AND METHODS

Participants

This retrospective clinical study included 776 patients with lung cancer and 794 controls who visited Meizhou *People's Hospital (Huangtang Hospital),* Meizhou Academy of Medical Sciences, *Meizhou* Hospital Affiliated to Sun Yat-sen University between January 2017 and September 2018. Informed consent was obtained from all patients. The study was conducted on the basis of the Declaration of Helsinki and was supported by the Ethics Committee of the Meizhou *People's Hospital*.

Sample preparation and tumor marker detection

Three milliliters of blood sample for tumor marker testing was obtained from each subject. Serum was separated and stored at -80°C until further analysis. CEA, CA15-3, CYFRA21-1, NSE, and SCCA were measured routinely by flow fluorescence method with the Quantitative Detection Kit for Tumor Markers (Tellgen Life Science, Shanghai, China). Critical values of CEA, CA15-3, CYFRA21-1, NSE, and SCCA were 5.00 ng/mL, 28.00 U/mL, 5.00 ng/mL, 25.00 ng/mL, and 1.50 ng/mL, respectively. For the combination of these markers, the judgment is positive when any one marker is positive, and the judgment is generally negative when all markers were negative.

DNA extraction and detection of *EGFR* gene mutations by ARMS PCR

After formalin-fixed and paraffin-embedded slices were deparaffinized, DNA was extracted by AmoyDx® Tis-

sue DNA Kit (Spin Column) (Amoy Diagnostics, Xiamen, China), following the manufacturer's instructions and NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to evaluate the quantity and quality of extracted DNA. The extracted DNA was stored at -20°C until use.

Amplification refractory mutation system (ARMS) is a highly sensitive real-time PCR-based test that uses the principle of ARMS and covers the 29 mutation hotspots from exon 18 to 21 in the *EGFR* gene. The assay was carried out according to the manufacturer's protocol for the *EGFR* Gene Mutations Fluorescence Polymerase Chain Reaction Diagnostic Kit (Amoy Diagnostics, Xiamen, China) with the LightCycler 480 real-time PCR system (Roche Diagnostics, Germany). Each PCR contained 5 - 10 pmol specific primers, 20 pmol double-loop probes, 12.5 µmol dNTPs, 175 µmol magnesium chloride, 1 mmol ammonium sulfate, and 2.5 mmol potassium chloride. PCR was performed with initial denaturation at 95°C for 5 minutes, followed by 15 cycles of first amplification (at 95°C for 25 seconds, 64°C for 20 seconds, and 72°C for 20 seconds) and 31 cycles of second amplification (at 95°C for 25 seconds, 60°C for 35 seconds, and 72°C for 20 seconds). The results were analyzed according to the criteria defined by the manufacturer's instructions. Positive results were defined as Ct (sample) - Ct (control) < Ct (cutoff).

Statistical analysis

SPSS statistical software version 21.0 was used for data analysis. The levels of tumor markers were expressed as the means ± SD. The rank sum test was used to compare the levels of tumor markers in each group and the chi-square (χ^2) test was used to compare the rates or ratios. To compare the diagnostic accuracy of detection markers in predicting pathological type of lung cancer, receiver operator characteristic (ROC) curves were generated and the area under the curve was calculated. A value of $p < 0.05$ was considered as statistically significant.

RESULTS

Population characteristics

A total of 1,570 patients (1,144 males and 426 females) were recruited in the study, and age was 59.01 ± 13.38 years in all subjects, of which for the males it was 59.60 ± 12.93 years and 57.43 ± 14.40 years for the females. There were 776 patients (59.78 ± 10.39 years) with lung cancer and 794 (58.26 ± 15.73 years) as controls. There were 537 (69.20%) patients with lung adenocarcinomas, 150 (19.33%) with squamous cell carcinomas, and 89 (11.47%) with small cell lung cancer. There were 113 (14.56%) patients with stages I/II, 148 (19.07%) constituent with stage III, and 515 (66.37%) with stage IV according to TNM classification of malignant tumors. The clinical characteristics of the subjects, including the immunohistochemistry status (CK7, TTF-1, Napsin-A, CD56, CK5/6 and CgA) in this study are presented in Table 1.

Comparison of the CEA, CA15-3, CYFRA21-1, NSE, and SCCA concentrations in the lung cancer group and control group, squamous cell lung cancer, small cell lung cancer, and lung adenocarcinoma

Comparison of the CEA, CA15-3, CYFRA21-1, NSE, and SCCA concentrations among groups, showed that the levels of CEA ($p < 0.001$), CA15-3 ($p = 0.001$), CYFRA21-1 ($p < 0.001$), and NSE ($p < 0.001$) in the lung cancer group was significantly higher than the control group, while the levels of CEA ($p < 0.001$) and CA15-3 ($p = 0.002$) in lung adenocarcinoma was observably higher than that in squamous cell lung cancer and small cell lung cancer. The levels of CYFRA21-1 ($p < 0.001$) and SCCA ($p < 0.001$) in serum of the lung squamous cell carcinoma patients were significantly higher than in the lung adenocarcinoma patients and small cell carcinoma patients, but there was no significant difference in serum SCCA levels between the lung cancer group and control group ($p = 0.340$). The level of NSE ($p < 0.001$) in the small cell lung cancer patients was significantly higher than that in the lung adenocarcinoma patients and squamous cell lung cancer patients, indicating that NSE is an important indicator for clinical diagnosis of small cell lung cancer. Detailed data are presented in Table 2.

Specificity and sensitivity of individual tumor markers and in the combination of these markers for lung cancer

In this study, tumor markers were detected in lung cancer patients and controls. Individual sensitivity of these tumor markers sorted in descending order were CEA (45.10%) > CYFRA21-1 (39.69%) > CA15-3 (37.50%) > NSE (19.85%). The combination of tumor markers can improve the sensitivity, and the combination of CEA + CA15-3 + CYFRA21-1 + NSE ranked the highest in the sensitivity index (75.00%) in lung cancer. The individual specificity of tumor markers were NSE (86.02%) > CYFRA21-1 (83.25%) > CEA (81.99%) > CA15-3 (69.14%). Specificity can be improved by combining tumor markers, and the combination of CEA + CA15-3 + CYFRA21-1 + NSE ranked the highest in the specificity index (98.61%) in lung cancer (Table 3).

Comparison on ROC curves of CEA, CA15-3, CYFRA21-1, and NSE

Comparison the accuracies when using CEA, CA15-3, NSE, and CYFRA21-1 in the diagnosis of different types of lung cancer is done by calculating the area under the ROC curve (AUROC). In lung cancer, the AUC of CEA (0.643; 95% CI: 0.615 - 0.670; $p < 0.001$) and CYFRA21-1 (0.640; 95% CI: 0.613 - 0.668; $p < 0.001$) were higher than those of CA15-3 (0.544; 95% CI: 0.515 - 0.572; $p = 0.003$) and NSE (0.533; 95% CI: 0.504 - 0.561; $p = 0.026$) (Figure 1A). In adenocarcinoma, the AUC of CEA (0.665; 95% CI: 0.634 - 0.697;

Table 1. Baseline characteristics of subjects.

	Lung cancer (n = 776)	Controls (n = 794)	p-value
Gender (male), n (%)	532 (68.56)	612 (77.08)	< 0.001
Age, mean ± SD (IQR)	59.78 ± 10.39 (20 - 97)	58.26 ± 15.73 (6 - 96)	0.025
Histology (ADK), n (%)			
Adenocarcinoma, n (%)	537 (69.20)		
Squamous cell lung cancer, n (%)	150 (19.33)		
Small cell lung cancer, n (%)	89 (11.47)		
NSCLC TNM stage			
I/II, n (%)	113 (14.56)		
III, n (%)	148 (19.07)		
IV, n (%)	515 (66.37)		
Immunohistochemistry			
CK7(+)	67/87		
TTF-1(+)	153/221		
Napsin-A(+)	54/118		
CD56(+)	45/76		
CK5/6(+)	42/103		
CgA(+)	35/54		

IQR - interquartile range, ADK - adenocarcinomas. TNM, TNM classification of malignant tumors (by meticulous description of the primary tumor (T), related lymph nodes (N) and any discernible metastases (M) it is possible to analyze groups of patients in many different ways).

Table 2. Different levels of tumor markers were observed among groups.

	CEA	CA15-3	CYFRA21	NSE	SCC
Lung cancer	23.57 ± 57.39	47.37 ± 85.12	10.74 ± 22.54	22.13 ± 22.22	0.95 ± 3.07
Controls	5.78 ± 22.56	35.03 ± 61.66	4.10 ± 6.37	18.32 ± 11.79	0.82 ± 2.20
p-value	< 0.001	0.001	< 0.001	< 0.001	0.340
Groups according to histology					
Lung adenocarcinoma	30.26 ± 67.00	54.33 ± 96.78	10.16 ± 22.23	19.27 ± 14.97	0.63 ± 1.91
Squamous cell lung cancer	7.60 ± 16.62	34.47 ± 49.71	16.97 ± 28.16	20.08 ± 15.97	2.31 ± 5.61
Small cell lung cancer	10.08 ± 17.35	27.12 ± 39.69	3.72 ± 2.39	42.79 ± 45.41	0.59 ± 1.83
p-value	< 0.001	0.002	< 0.001	< 0.001	< 0.001

$p < 0.001$) and CYFRA21-1 (0.631; 95% CI: 0.600 - 0.662; $p < 0.001$) were higher than those of CA15-3 (0.559; 95% CI: 0.527 - 0.591; $p < 0.001$) and NSE (0.507; 95% CI: 0.475 - 0.539; $p = 0.666$) (Figure 1B). In squamous carcinoma, the AUC of CYFRA21-1 (0.722; 95% CI: 0.668 - 0.776; $p < 0.001$) and SCC (0.628; 95% CI: 0.573 - 0.683; $p < 0.001$) were higher than those of CEA (0.579; 95% CI: 0.526 - 0.632; $p = 0.002$), CA15-3 (0.524; 95% CI: 0.473 - 0.575; $p = 0.347$) and NSE (0.552; 95% CI: 0.500 - 0.604; $p = 0.043$) (Figure 1C). In small cell carcinoma, the AUC of NSE (0.654; 95% CI: 0.576 - 0.732; $p < 0.001$) was higher than those of CEA (0.616; 95% CI: 0.549 - 0.683; $p < 0.001$), CYFRA21-1 (0.555; 95% CI: 0.489 - 0.622; $p = 0.087$) and CA15-3 (0.482; 95% CI: 0.421 - 0.543; $p = 0.579$) (Figure 1D). Detailed data are presented in Table 4 and Figure 1.

Table 3. Sensitivity and specificity of serum tumor markers for lung cancer.

Tumor markers	Sensitivity (%)	Specificity (%)	Youden's index
CEA	45.10 (350/776)	81.99 (651/794)	0.27
CA15-3	37.50 (291/776)	69.14 (549/794)	0.07
CYFRA21-1	39.69 (308/776)	83.25 (661/794)	0.23
NSE	19.85 (154/776)	86.02 (683/794)	0.06
CEA + CA15-3	63.02 (489/776)	94.96 (754/794)	0.58
CEA + CYFRA21-1	63.79 (495/776)	92.70 (736/794)	0.56
CEA + NSE	53.74 (417/776)	95.84 (761/794)	0.50
CA15-3 + CYFRA21-1	58.63 (455/776)	93.83 (745/794)	0.52
CA15-3 + NSE	48.07 (373/776)	92.32 (733/794)	0.40
CYFRA21-1 + NSE	46.39 (360/776)	94.84 (753/794)	0.41
CEA + CA15-3 + CYFRA21-1	73.20 (568/776)	97.23 (772/794)	0.70
CEA + CA15-3 + NSE	66.88 (519/776)	98.11 (779/794)	0.65
CEA + CYFRA21-1 + NSE	67.53 (524/776)	96.98 (770/794)	0.65
CA15-3 + CYFRA21-1 + NSE	62.24 (483/776)	97.73 (776/794)	0.60
CEA + CA15-3 + CYFRA21-1 + NSE	75.00 (582/776)	98.61 (783/794)	0.74

Table 4. Areas under the ROC curve and predictive value of four tumor markers for lung cancer.

	CEA	CA15-3	CYFRA21	NSE
95% CI	0.615 - 0.670	0.515 - 0.572	0.613 - 0.668	0.504 - 0.561
p-value	< 0.001	0.003	< 0.001	0.026
AUC	0.643	0.544	0.640	0.533
Cutoff point	5.310	20.325	4.115	19.425
Specificity (%)	44.3	50.1	46.8	35.6
Sensitivity (%)	83.9	60.2	78.1	74.6

95% CI, 95% confidence interval, AUC - area under the ROC curve.

The frequencies and distributions of EGFR gene mutation

In NSCLC patients, the *EGFR* gene was detected in 208 patients. Of those, 91 (43.75%) with *EGFR* gene mutations included 47 (22.60%) with the 19-del mutation, 33 (15.86%) with the L858R mutation, 2 (0.96%) with the 20-Ins mutation, 1 (0.48%) with the G719X and L861Q mutations, 3 (1.44%) with the T790M spontaneous mutation, and the T790M resistance mutation occurred in 4 patients (1.92%) after TKI targeted therapy.

Thirty patients with *EGFR* sensitive mutations were treated with Gefitinib, 20 with Icotinib and 1 with Erlotinib. Three patients with the *EGFR* sensitive mutations chose to give up treatment, while the others chose conventional chemotherapy. One patient with *EGFR* T790M mutation was treated with conventional chemotherapy, while the others were treated with osimertinib.

DISCUSSION

In the process of growth and proliferation, the tumor exchanges substances with the surrounding environment constantly, and these substances are not only necessary for the tumor cell growth, invasion, and metastasis. The tumor secreting proteins can be used as tumor markers, and detection of these markers in the body fluids such as blood is a quick and easy method. It is particularly important in the early tumor detection. In clinical practice, CEA, CA15-3, CYFRA21-1, NSE, and SCCA are the tumor markers most widely used for the diagnosis of lung cancer.

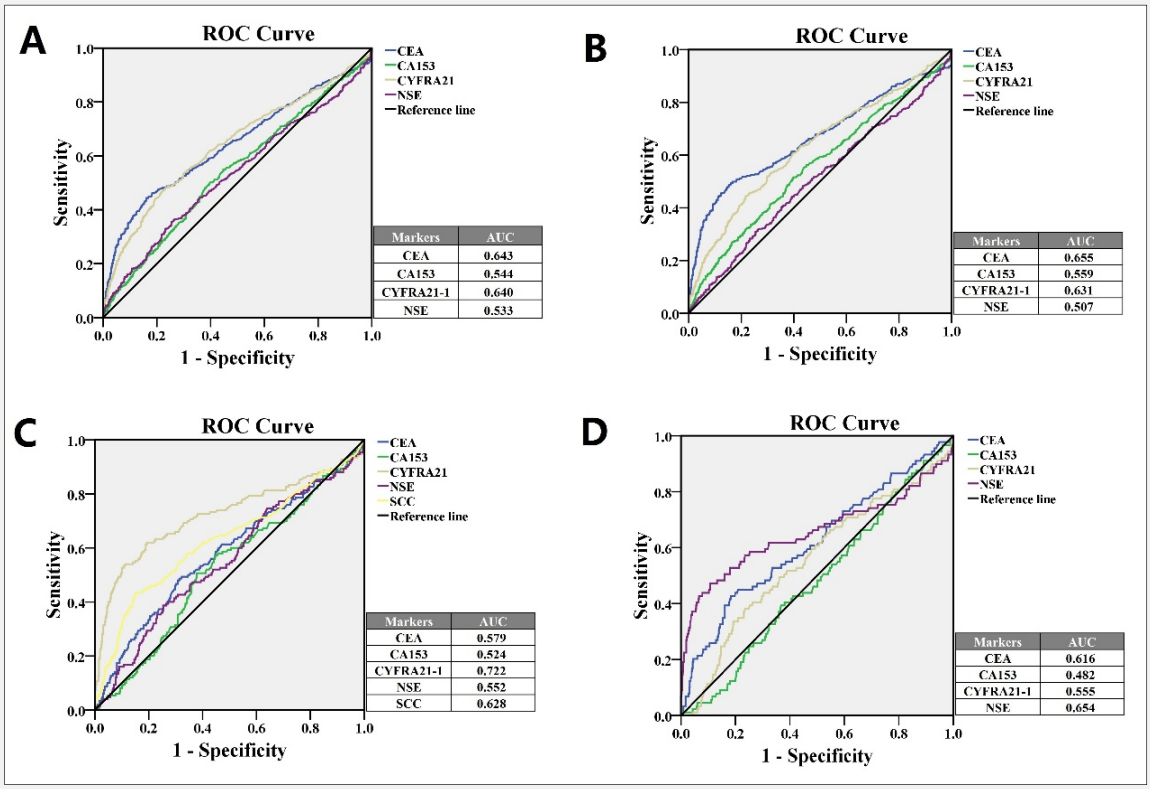

Figure 1. ROC Curves of CEA, CA15-3, CYFRA21, and NSE for lung cancer.

A - lung cancer, B - adenocarcinoma (AC), C - squamous carcinoma (SC), D - small cell carcinoma (SCC).

CEA was first described as an antigen expressed by gastrointestinal cancer cells and is a non-organ specific tumor-related antigen. The increase of CEA level in the circulating blood is closely related to the onset and progression of cancer and is widely used in the detection of various types of tumors [22,23]. This study indicated that the level of CEA in the lung adenocarcinoma patients group was obviously higher than that in the squamous cell lung cancer group and small cell lung cancer group. CEA has high specificity (81.99%) in the diagnosis of lung cancer in this study. CEA is the most sensitive of these tumor markers in the diagnosis of lung cancer.

Cancer antigen 15-3 (CA 15-3) is an antibody produced by normal cells. In many patients with cancers, there is an increased production and release of CA15-3 by the tumor cells. As it enters the bloodstream, the determination in blood makes it useful as a tumor marker to follow the course of the cancer [15]. CA15-3 may be elevated in individuals with some cancers, such as lung cancer and breast cancer. This study indicated that the level of CA15-3 in the lung cancer group was obviously higher than in the control group, while the lung adenocarcinoma group was obviously higher than that in the squamous cell lung cancer group and small cell lung cancer group.

CYFRA21-1, a fragment of cytokeratin 19, is mainly distributed in tumor cells originating from epithelial cells and the level of CYFRA21-1 is elevated in serum in lung cancer patients. The results of the present study showed that the level of CYFRA21-1 in the lung cancer group was obviously higher than that in the control group. According to the serum level and the ROC curves shown in this study, serum CYFRA21-1 level of lung squamous cell carcinomas patients was significantly higher than lung adenocarcinoma patients and small cell carcinoma patients. Our results verified CYFRA21-1 is an important marker for squamous cell carcinoma diagnosis [24-26].

NSE is the isoenzyme of neuron-specific enolase and can be used as a specific tumor marker because it is overexpressed in small-cell lung cancer [27,28]. The result of this study indicated that NSE is a valuable indicator for the diagnosis of small cell lung cancer [18,28].

NSE is one of the most specific tumor markers in the diagnosis of lung cancer.

SCCA is a serological tumor marker and it is a subfraction of the tumor-associated antigen TA-4, encoded by SCCA1 and SCCA2 [29-32]. SCCA expression increases in all squamous cell carcinomas including uterine cervix, lung, head and neck, esophagus, and anal canal, as well as in several non-malignant skin disorders and renal failure [33,34]. In this study, although there was no obvious difference in serum SCCA levels between lung cancer group and control group ($p = 0.340$), squamous cell lung cancer patients were obviously higher than that in lung adenocarcinoma and small cell lung cancer patients, indicating that SCCA is an important serum marker for the diagnosis of squamous cell lung cancer.

In this study, five common serum markers (CEA, CA15-3, CYFRA21-1, NSE, and SCCA) of lung cancer were evaluated separately and jointly. On the basis of sensitivity and specificity, the ROC curve and Youden's index were used for verification, and the test results were more accurate, effective, and comprehensive. Compared to CA15-3 and NSE, CEA and CYFRA21-1 were more accurate because they had higher Youden's indexes (0.27 and 0.23, respectively) and larger areas under ROC curves (0.643 and 0.640, respectively). However, the sensitivities of 4 individual markers were lower than 50%. Our study shows that the sensitivities of tumor markers can be improved by a combination of these markers. The combination of CEA + CA15-3 + CYFRA21-1 + NSE might be an optimal choice according to the higher specificity (98.61%) and sensitivity (75.00%) with the highest Youden's index (0.74).

In this study, the *EGFR* gene was detected in 208 patients with non-small cell lung cancer. We are following up these patients who have been treated with tyrosine kinase inhibitors and testing the level of tumor markers during their treatment. Follow-up and monitoring of the efficacy of these patients is the focus of our next work. In the next study, we will also continue to include more research subjects and enrich relevant data. In conclusion, the results suggest that among the chosen markers, combined measurement of CEA, CA15-3, CYFRA21-1, NSE, and SCCA in serum is useful in the diagnosis of lung cancer. At the same time, we need to find more specific and sensitive markers for early diagnosis, therapeutic evaluation, and prognosis of lung cancer.

CONCLUSION

Lung cancer is the most common and fatal malignant tumor in most of countries and it is becoming one of the major health problems in some developing countries. The above serum tumor markers are valuable indicators in clinical use. Although these markers in our study are quick, objective, comparable, and reproducible, there is no single serum marker sensitive enough for diagnosis and screening of lung cancer. Combined with tumor markers, they can be used to improve the clinical efficacy of lung cancer diagnosis. New specific and sensitive markers for early diagnosis, therapeutic evaluation, and prognosis of lung cancer also needed.

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Ethics approval and consent to participate:
The study was approved by the Ethics Committee of Medicine, Meizhou People's Hospital (Huangtang Hospital), Meizhou Academy of Medical Sciences, Meizhou Hospital Affiliated to Sun Yat-sen University.

Availability of data and materials:
The data used to support the findings of this study are available from the corresponding author upon request.

Authors' Contributions:
Heming Wu and Qiuming Wang designed the study. Qunji Zhang, Qingyan Huang, and Zhikang Yu performed the experiments. Heming Wu and Qiuming Wang collected clinical data. Heming Wu and Qiuming Wang analyzed the data. Heming Wu prepared the manuscript. All authors were responsible for critical revisions, and all authors read and approved the final version of this work.

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Declaration of Interest:
The authors declare that they have no competing interests with the contents of this paper.

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