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Identification of plasma SAA2 as a candidate biomarker for the detection and surveillance of non-small cell lung cancer

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This study aimed to measure the expression of SAA2 in plasma and to assess its diagnostic efficacy as a biomarker for non-small cell lung cancer (NSCLC). The gene expression of SAA2 in NSCLC was analyzed based on a database. Then, SAA2 expression was detected by immunohistochemistry in lung tissue and by enzyme-linked immunosorbent assay in 90 patients with NSCLC and 61 normal controls. Finally, the diagnostic performance was assessed in terms of accuracy, sensitivity, and specificity. At the gene and protein levels, the SAA2 expression was significantly higher in the NSCLC group than in the control group ($p<0.01$). It was higher in lung squamous carcinoma than in lung adenocarcinoma and in males than in females, and this trend was also observed in the lung squamous carcinoma group. Of note, the expression of SAA2 increased with increasing disease stage. Receiver operating characteristic (ROC) curve analysis revealed that the sensitivity of SAA2 was 83.61%, the specificity was 91.11%, and the area under the curve (AUC) was 0.9252. Its accuracy was 68.89%, which was higher than that of other conventional diagnostic biomarkers, and the combined application can effectively improve the diagnostic efficiency. Based on the results, SAA2 expression was positively correlated with the disease stage of NSCLC. Notably, SAA2 is more concerning in male patients with lung squamous carcinoma, and it can help in the screening and diagnosis of NSCLC. SAA2 may represent a novel diagnostic biomarker in NSCLC.

Key words: non-small cell lung cancer, biomarkers, SAA2, diagnosis, immunohistochemistry

Lung cancer is the leading cause of cancer deaths worldwide because it is initially asymptomatic but usually discovered at advanced stages(Wang, Hao, Pan, Qian, & Zhou, 2015). According to the World Health Organization (WHO), lung cancer became the most common cancer and had the highest morbidity and mortality in 2018 [1]. In total, 2,813,503 deaths were recorded in the United States in 2018, 21% of which were from cancer [2]. Among men, lung cancer remains the most common cancer diagnosis, with approximately 116,000 cases in 2020. Among women, the incidence rates are generally lower than those of men, with over 112,000 new lung cancer diagnoses in 2020 [3]. In 2015, the incidence of lung cancer in China was approximately 730,000 including 510,000 men and 224,000 women, and it has still been increasing in recent years [4]. The early identification of lung cancer can effectively reduce mortality and improve treatment outcomes [5].

Electromagnetic navigation bronchoscopy is a helpful tool to sensitively identify lung cancer [6]. The combination of bronchial genomic classifier and bronchoscopy can improve the diagnostic sensitivity of lung cancer [7]. The national lung screening test (NLST) of the US shows that low-dose computed tomography (LDCT) can efficiently screen lung cancer patients and reduce the mortality of lung cancer [8]. However, it can also lead to many false-positive results and requires radiation. Biopsies can obtain detailed and accurate data. However, its deficiency lies in the discomfort caused by highly invasive procedures, in addition to the time-consuming nature of the process [9]. Despite these approaches, the adoption of lung cancer screening remains insufficient due to the high number of false positives or invasiveness of the procedures. Therefore, developing new blood markers can improve the implementation of lung cancer screening and serve as an important supplement to routine examination

[10]. Tumor-derived components from blood samples can be used as biomarkers for lung cancer screening and for the classification of uncertain lung nodules [11]. Peripheral blood biomarkers have the advantages of noninvasiveness, convenient collection, and low cost. Therefore, the analysis of peripheral blood biomarkers is a new method widely used to detect primary lung cancer [12]. Quantitative proteomics has become the dominant means of discovering new potential cancer biomarkers in serum/plasma, which can be used in clinical diagnosis, prognosis, and prediction [13]. Some plasma biomarkers, such as sP-selectin and fibrinogen, are strongly positively related to lung cancer and could indicate a higher risk of lung cancer [14]. However, the existing biomarkers are often not comprehensive and lack specificity. Therefore, new plasma biomarkers for lung cancer need to be identified.

As a normal component of serum, serum amyloid protein A (SAA) is small and mainly synthesized in the liver [15]. It is secreted outside the cells and is mainly involved in the activation of chemokines. The plasma level of SAA protein increases sharply after trauma, infection, and other stimulation. The SAA protein is a molecular family that includes two members, SAA1 and SAA2. Isotype-specific parallel reaction monitoring (PRM) detection can be used to distinguish different types of SAAs, but the relationship between SAA2 and lung cancer is still unclear [16].

In the present work, SAA2 was identified as a candidate biomarker for non-small cell lung cancer (NSCLC) from differentially expressed genomics and proteomics databases. Then, the diagnostic performance of SAA2 in NSCLC was analyzed by immunohistochemistry (IHC) and enzyme-linked immunosorbent assay (ELISA). Finally, SAA2 was compared with other conventional tumor biomarkers, such as carcinoembryonic antigen (CEA), carbohydrate antigen 125 (CA125), carbohydrate antigen 19-9 (CA19-9), soluble fragment of cytokeratin 19 (CYFRA21-1), squamous cell carcinoma antigen (SCC), and neuron-specific enolase (NSE), and was identified as a potential supplementary diagnostic biomarker for lung cancer.

Patients and methods

Clinical samples. Paraffin sections of lung cancer and adjacent tissues were collected from Shanghai Public Health Clinical Center, and their pathological types were identified by specialists. The plasma of 90 patients with lung cancer and 61 normal volunteers was collected from Renji Hospital Affiliated with Shanghai Jiao Tong University School of Medicine. Human peripheral blood samples for ELISA analysis were collected and centrifuged at 4 °C at 1600×g for 15 min within two hours and then transferred to a new Eppendorf tube to separate and collect the supernatant plasma. All plasma tubes were marked and immediately stored at –80 °C.

Clinical information collection. Cases expressing one or more of six clinical biomarkers were collected from Renji Hospital Affiliated with Shanghai Jiao Tong University School of Medicine, including 84 cases expressing CEA; 78 cases expressing CA125; 76 cases expressing CA19-9; 76 cases expressing CYFRA21-1; 83 cases expressing SCC and 82 cases expressing NSE. In addition, from the database of

Table 1. Basic data of patients with lung cancer and healthy controls.

Variable	Number	Variable		Number
Lung cancer	90	Normal control		61
LUAD	62	LUSC		28
LUAD males	38	LUAD females		24
LUSC males	28	LUSC males		0
Stage I males	13	Stage I females		13
Stage II males	9	Stage II females		1
Stage III males	24	Stage III females		2
Stage IV females	20	Stage IV females		8
Received treatment	18	Did not receive treatment		72
Smoking	1			
Pathological paraffin sections (cancer)	15	Pathological paraffin sections (adjacent)		15
Genetic databases (cancer)	969	Genetic databases (normal)		109
Protein databases (Differential upregulation of expression)	148	Protein databases (Differential downregulation of expression)		129
Other biomarkers information (Number of cases)	CEA	84	CA125	78
	CA19-9	76	CYFRA21-1	76
	SCC	83	NSE	82

Abbreviations: NSCLC-non-small cell lung cancer; LUAD-lung adenocarcinoma; LUSC-lung squamous cell carcinoma; CEA-carcinoembryonic antigen; CA125-carbohydrate antigen 125; CA19-9-carbohydrate antigen 19-9; CYFRA21-1-soluble fragment of cytokeratin 19; SCC-squamous cell carcinoma antigen; NSE-neuron-specific enolase;
Notes: Treated-chemotherapy, radiotherapy, or surgery; Untreated-initial diagnosis

differentially expressed proteins in human cancer (dbDEPC; https://www.scbit.org/dbdepc3/index.php), 227 differentially expressed proteins in lung cancer were obtained, of which 148 were upregulated and 129 were downregulated. SAA2 was selected among the upregulated proteins. Moreover, the clinical data of 1,078 lung cancer patients were provided by Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/), which shows that SAA2 is upregulated at the gene level.

IHC. Paraffin sections from the NSCLC and normal groups were deparaffinized in xylene and rehydrated in a descending ethanol series (100%, 95%, 90%, 80%, and 70% ethanol) and double-distilled water according to standard protocols. Heat-induced antigen retrieval was performed in citrate buffer, and the sections were boiled for 15 min. After antigen retrieval, the sections were treated with 3% hydrogen peroxide to block endogenous peroxidase activity and nonspecific binding. The sections were incubated with SAA2 antibody (KANGLANG, Cat. No. KL-80953-01 (100 µl), dilution 1:100) at room temperature for one hour and washed with phosphate-buffered saline. The tissue sections were incubated with goat anti-rabbit enzyme-labeled secondary antibody (Thermo, diluted 1:200) for one hour at room temperature. Diaminobenzidine was used as the chromogen, and the tissue sections were counterstained with hematoxylin and then viewed under a bright-field microscope.

ELISA. According to the manufacturer's instructions (Cloud-Clone Corp, Wuhan, China), a commercial sandwich ELISA kit was used for the serological measurement of SAA2. All ELISA measurements were repeated at least once for each sample. The detail of the ELISA kit as follows:

Enzyme-linked Immunosorbent Assay Kit for Serum Amyloid A2 (SAA2). Brand: Cloud-Clone Corp, Wuhan, China. Cat. Number: SEB795Hu 96 Tests. Detection range: 6.25-400 ng/ml. Sensitivity: The minimum detectable dose of SAA2 is typically less than 2.64. Specificity: This assay has high sensitivity and excellent specificity for the detection of SAA2. No significant cross-reactivity or interference between SAA2 and analogs was observed. Precision: Intra-assay Precision (Precision within an assay): 3 samples with low, middle, and high-level SAA2 were tested 20 times on one plate, respectively. Inter-assay Precision (Precision between assays): 3 samples with low, middle, and high-level SAA2 were tested on 3 different plates, with 8 replicates in each plate. CV (%) = SD/mean × 100; intra-Assay: CV <10%; inter-assay: CV <12%. Stability: The stability of the ELISA kit is determined by the loss rate of activity. The loss rate of this kit is <5% prior to the expiration date under appropriate storage conditions. It should be noted that our plasma samples were diluted 10 times for testing.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA), and significance was determined using Student's t-test. The screening of differentially expressed genes and proteins was conducted online. The two-tailed t-test was used to detect differences between the two groups. One-way analysis of variance (ANOVA) was used to test for differences among three or more groups, with $p<0.05$ indicating that the difference was statistically significant.

Results

Descriptive statistics. A total of 151 plasma samples were collected from patients aged between 22 and 79 years; 90 of these patients were diagnosed with NSCLC. Some detailed information is displayed below (Table 1).

Gene expression and proteomic data of SAA2 in lung cancer. Compared with the normal group, SAA2 was differentially expressed in lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) at the gene level (Figure 1A). The expression of the SAA2 gene in 483 lung cancer and 59 normal tissues was analyzed (based on transcripts per kilobase million (TPM)). Compared with the normal group, SAA2 was significantly upregulated in LUAD ($p<0.01$, Figure 1B). From the proteomics database, we found that SAA2 is an upregulated protein in LUAD (Table 2). At both the gene and protein levels, SAA2 is significantly upregulated in lung cancer.

Semiquantitative determination of SAA2 by IHC. Visually, SAA2 is expressed in the extracellular cytoplasm, especially around the cavity of the tissue section (Figure 2). The pathological sections of 6 patients enrolled from the clinic

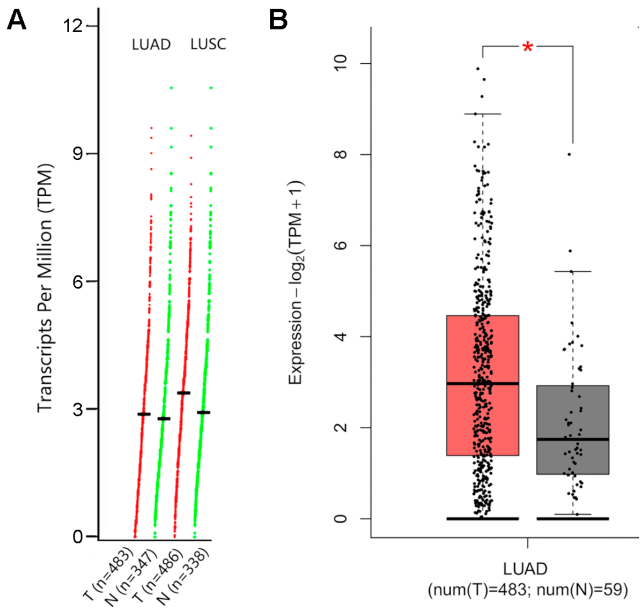

Figure 1. Expression of SAA2 at the gene level. The horizontal axis is the normal or lung cancer group, and the vertical axis indicates the expression level of SAA2. A) Expression level of SAA2 in LUAD, LUSC, and tumor-adjacent normal tissues. B) Differential expression of SAA2 in LUAD tissues, which is marked with a red asterisk (*p<0.05). Abbreviations: TPM-transcripts per kilobase million; LUAD-lung adenocarcinoma; LUSC-lung squamous cell carcinoma; T-tumor; N-normal

Figure 2. Representative images of immunohistochemical staining of SAA2 in tumor tissues. A, C, E) NSCLC cancer tissues (Mi); B, D, F) matched tumor-adjacent normal tissues of A, C, and E (nMi). (DAB staining and SP method; magnification, ×200). Abbreviations: nMi-non-miliary; Mi-miliary; SP-Streptomyces; DAB-3,3'-diaminobenzidine. Lung cancer tissues were deeply stained, while paracancerous tissues were almost unstained, and almost no large extracellular cytoplasm or tissue lacunae could be observed.

Table 2. SAA2 is a differentially expressed upregulated protein in the dbDEPC database.

EXPID	Cancer ID	UniProt KB	Cancer name	Gene name	Length	Differential expression
EXP00154	C00001	Q12805	Lung Adenocarcinoma	SAA2	122	up

Note: according to the database; SAA2 is one of the differentially upregulated proteins in NSCLC

were analyzed by special IHC analysis software. Compared with that in the paracancerous group, the expression of SAA2 was significantly higher in the carcinoma group, as shown in Tables 3 and 4 and Figures 3 and 4.

SAA2 expression level and its diagnostic performance. ELISA showed that SAA2 was significantly upregulated in the lung cancer group compared with the normal group (Figure 5A). Compared with the normal group, SAA2 was significantly upregulated in the LUAD group and the LUSC group; among them, SAA2 expression was higher in LUSC than in LUAD (Figure 5B), which is consistent with the gene expression of SAA2 in the gene database (Figure 1). In addition, SAA2 was higher in males than in females (Figure 5C), and this trend was maintained in LUSC (Figure 5D). Moreover, it is noteworthy that the expression of SAA2 increased with increasing disease stage (Figure 5E).

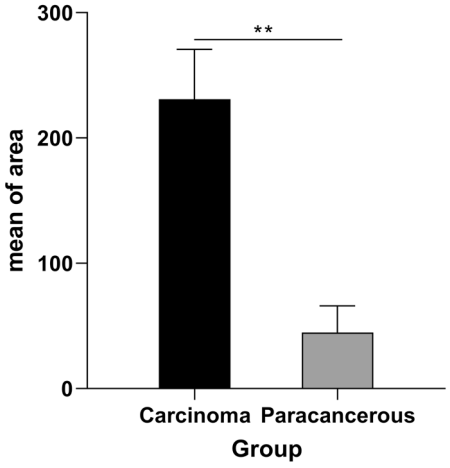

Figure 3. Comparison of the mean staining area between cancer and paracancerous groups. The staining area in the carcinoma group was 230.90±39.77, while that in the paracancerous group was 44.87±22.05, **p<0.002.

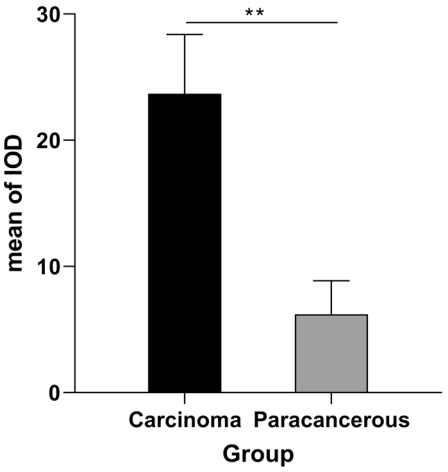

Figure 4. Comparison of the mean staining area between cancer and paracancerous groups. The mean IOD in the carcinoma group was 23.68±4.71, while that in the paracancerous group was 6.22±2.64, **p<0.005.

Table 3. Mean positive staining area of pathological sections.

Section	Carcinoma	Section	Paracancerous
a	196.55	b	34.25
c	221.67	d	69.12
e	274.46	f	31.24

Note: in this table, a, c, and e are cancerous tissues and b, d, and f are adjacent tissues

Table 4. Mean of IOD of pathological sections.

Section	Carcinoma	Section	Paracancerous
a	18.95	b	5.26
c	28.37	d	9.21
e	23.73	f	4.19

Note: in this table, a, c, and e are cancerous tissues and b, d, and f are adjacent tissues

Table 5. ROC curve analysis of biomarkers.

Biomarker	Specificity %	Sensitivity %	AUC
SAA2	91.11	83.61	0.9252
CEA	67.86	78.57	0.7756
CA125	56.41	98.72	0.7189
CA19-9	55.26	100	0.7774
CYFRA21-1	76.32	100	0.8901
SCC	50.60	100	0.7071
NSE	46.34	100	0.6907

Abbreviations: CEA-carcinoembryonic antigen; CA125-carbohydrate antigen 125; CA19-9-carbohydrate antigen 19-9; CYFRA21-1-soluble fragment of cytokeratin 19; SCC-squamous cell carcinoma antigen; NSE-neuron-specific enolase; ROC-receiver operator characteristic; AUC-area under the curve

Table 6. Comparison of the diagnostic accuracy of different biomarkers.

Biomarker	SAA2	CEA	CA125	CA19-9	CYFRA21-1	SCC	NSE
Total number of cases	62	28	18	9	35	13	10
Hit numbers	90	84	78	76	76	83	82
Accuracy %	68.89	33.33	23.07	11.84	46.05	15.66	12.20

Abbreviations: CEA-carcinoembryonic antigen; CA125-carbohydrate antigen 125; CA19-9-carbohydrate antigen 19-9; CYFRA21-1-soluble fragment of cytokeratin 19; SCC-squamous cell carcinoma antigen; NSE-neuron-specific enolase

Then, receiver operating characteristic (ROC) curve analysis showed that SAA2 had the best diagnostic performance. The details are shown in Figure 5F and Table 5. In addition, the accuracy of SAA2 was higher than that of other conventional clinical diagnostic biomarkers, such as CEA, CA125, CA19-9, CYFRA21-1, SCC, and NSE (Figure 5G, Table 6).

In the ROC curves, we observed that CA125, CA19-9, and SCC were not completely above the diagonal. SAA2 combined with CEA, CYFRA21-1, and NSE was found to be beneficial in the diagnosis of NSCLC (Figure 5H, Table 7).

Discussion

Humoral immunity-based biomarkers can be developed as alternative means for early cancer diagnosis [17]. Prior studies have reported that SAA2 is related to immunity, inflammation, cancer, and other diseases. The SAA2 gene is

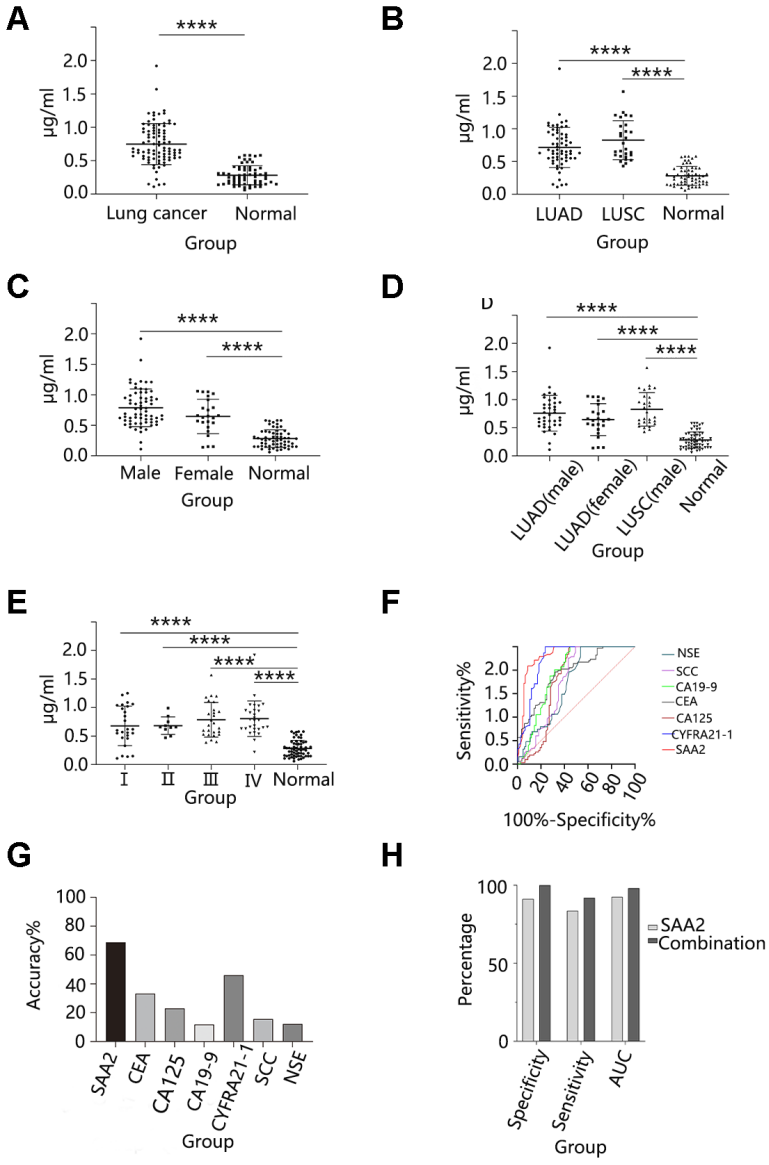

Figure 5. Verification of the SAA2 expression level by ELISA and its diagnostic performance (μg/ml). A) SAA2 was significantly upregulated in the lung cancer group compared with the normal group (p<0.0001; 0.75±0.31 vs. 0.28±0.14 μg/ml). B) Compared with the normal group, SAA2 was significantly upregulated in the LUSC group and the LUAD group (0.83±0.30 vs. 0.71±0.31 μg/ml). C) SAA2 was higher in the male group than in the female group of NSCLC patients (0.79±0.31 vs. 0.65±0.28 μg/ml). D) SAA2 was more highly expressed in the male group than in the female group of LUAD patients (0.76±0.32 vs. 0.65±0.28 μg/ml). E) SAA2 expression positively correlated with cancer stage in NSCLC (0.679±0.35 vs. 0.683±0.15 vs. 0.78±0.30 vs. 0.81±0.31 μg/ml). F) ROC curves of SAA2 and conventional markers. The horizontal axis represents the false-positive rate (100% – specificity %), and the vertical axis shows the true-positive rate (sensitivity); SAA2 had the best diagnostic performance. G) SAA2 has higher accuracy than other conventional biomarkers for NSCLC diagnosis. H) SAA2 combined with CEA, CYFRA21-1, and NSE was found to be beneficial in the diagnosis of NSCLC. Data are presented as the mean ± standard deviation (SD), ns: 0.1234, *p<0.0332, ****p<0.0001.

Table 7. Comparison of the diagnostic efficacy of SAA2 alone versus in combination.

Biomarker	Specificity %	Sensitivity %	AUC
SAA2	91.11	83.61	0.9252
Combination	100	91.90	0.9810

Note: combination - SAA2 combined with CEA, CYFRA21-1 and NSE
Abbreviations: SAA2-serum amyloid protein A2; AUC-area under the curve

expressed in human primary monocytes and macrophages [18] and plays a unique role in promoting Th17-mediated inflammatory diseases [19]. SAA2 has been found to be effective in distinguishing between infected and normal states and has the potential to be a biomarker for assessing the occurrence and development of bacterial bloodstream infections (BSIs) [20]. SAA2 was expressed at low levels

($p<0.05$) in patients with coronary artery disease (CAD) but highly expressed in the lung parenchyma of chronic obstructive pulmonary disease (COPD) patients [21]. SAA2 could be used as a potential marker for the diagnosis or treatment of ulcerative colitis (UC) and hepatocellular carcinoma (HCC) [22]. In the tumor microenvironment, SAA2 induces paracrine effects in various lung cancer models to stimulate migration, invasion, and metastasis [23].

Tumor molecular biomarkers serve as the basis of targeted therapy in the era of personalized drug therapy for advanced NSCLC [24]. Screening differentially expressed genes or proteins by genomics and proteomics has become a trend in lung cancer research. By searching databases, we found that SAA2 was differentially expressed in lung cancer at both the gene and protein levels. IHC and ELISA were conducted to verify the expression level of SAA2. The results showed that SAA2 was significantly upregulated in lung cancer (including LUAD and LUSC) compared to the normal group. Our experimental results showed that SAA2 has the best diagnostic efficacy when used alone, including high sensitivity and specificity and the highest accuracy. When combined with routine diagnostic markers such as CEA, CYFRA21-1, and NSE, the diagnostic effectiveness for NSCLC is further improved. Moreover, SAA2 was strongly correlated with the cancer type, sex, and cancer stage of NSCLC patients. It was especially highly expressed in male squamous carcinoma patients and positively related to the NSCLC stage.

The initial objective of the project was to identify the expression of SAA2 in lung cancer plasma and to assess its diagnostic value in NSCLC. We successfully found that the concentration of SAA2 in the plasma of NSCLC patients was approximately 2.5 times higher than that in the control group by ELISA, and SAA2 was found to have the highest accuracy among some diagnostic biomarkers for lung cancer. These results further provide evidence that SAA is highly expressed in cancer tissues [25]. The finding that SAA2 was significantly upregulated in LUAD and LUSC is somewhat surprising, given that the differentially expressed gene database shows that SAA2 is significantly upregulated in LUAD compared with the normal group. Another important finding was that SAA2 correlated with sex and NSCLC type, with the highest expression in males with LUSC. Furthermore, the higher the disease stage of cancer was, the higher the SAA2 expression. The results are inconsistent with those of Li et al. [26], who found that SAA2 was significantly upregulated in males but significantly downregulated in females. This unexpected finding might be explained by the fact that SAA2 expression is tumor type- and disease stage-dependent. Surprisingly, SAA2 was significantly highly expressed in LUSC, but all the patients were males, without our prior knowledge, and the samples were randomly selected. Perhaps SAA2 is only expressed in male LUSC patients, but the evidence is insufficient and warrants further validation.

The dynamic changes in CEA, CA125, and CYFRA21-1 relative to baseline have prognostic value for patients with advanced NSCLC treated with immunosuppressants [27]. For the diagnosis of lung cancer, although the specificities of CEA, NSE, and CYFRA21-1 are all 95%, their sensitivities are only 15.2%, 17.0%, and 17.9%, respectively [24], while SAA2 has the highest sensitivity (83.61%), outstanding specificity (91.11%), and a high area under the curve (AUC) (0.9252). Our data showed that the accuracy (46.05%) of CYFRA21-1 for NSCLC is the highest among the six routine diagnostic biomarkers. This seems to be consistent with other research that found that CYFRA21-1 was the most sensitive marker in NSCLC and that the serum level of CYFRA21-1 or SCC was significantly higher in squamous carcinoma ($p<0.05$) [28]. The serum levels of CYFRA21-1 and CEA can be used to distinguish between malignant and benign nodules [29]. A retrospective descriptive study showed that CEA, CYFRA21-1, and NSE were most commonly detected before the first diagnosis of NSCLC [30]. The serum levels of CEA, CA125, CYFRA21-1, and NSE were upregulated in NSCLC and were associated with the progression of NSCLC [31]. However, SAA2 has higher accuracy than CYFRA21-1.

The combination of CYFRA21-1, CA125, CEA, and other biomarkers has a high sensitivity for the early diagnosis of lung cancer [32], and the diagnostic value of the combination is significantly higher than that of each biomarker alone [33, 34]. However, our experiment also has shortcomings. We did not do multi-center and larger sample validation.

The present study indicates that SAA2 could serve as a potential clinical diagnostic biomarker for lung cancer. These encouraging results indicate that there is a certain correlation between SAA2 and NSCLC, and it contributes to the diagnosis of NSCLC. These findings suggest that the lowering of SAA2 may reduce hospital admissions for NSCLC.

In conclusion, our data indicate that SAA2 is highly differentially expressed in patients with NSCLC at both the gene and protein levels. SAA2 was positively correlated with disease stage and related to cancer type and patient sex. In particular, the significantly high expression of SAA is more concerning in male patients with LUSC. SAA2 has excellent accuracy, high sensitivity, and outstanding specificity compared with other conventional lung cancer diagnostic markers. The combination of SAA2 and other routine clinical biomarkers can effectively improve the diagnostic efficiency for NSCLC. Overall, the research presented here confirms that SAA2 could be utilized as a potential biomarker for the diagnosis of NSCLC and provides new ideas and entry points to resolve the low specificity and sensitivity of lung cancer-related tumor markers.

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