

Cancer testis antigen SPAG9 is a promising marker for the diagnosis and treatment of lung cancer

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Abstract. Cancer testis antigen sperm-associated antigen 9 (SPAG9) is highly expressed in many types of cancers. In the present study, to obtain a better understanding of the relevance of SPAG9 in cancer diagnosis and treatment, the expression of SPAG9 mRNA and protein in lung cancer specimens was evaluated by RT-PCR, western blotting and immunohistochemistry. ELISA was used to quantify the SPAG9 autoantibody in the peripheral blood of lung cancer patients. The results showed that the expression of SPAG9 mRNA and protein in the lung cancer tissues was significantly higher than that in the adjacent non-cancerous tissues ($P < 0.01$). The level of the SPAG9 autoantibody in the serum of lung cancer patients was significantly higher than the level in the healthy controls ($P < 0.001$), and the level of the SPAG9 autoantibody in the serum of untreated patients was significantly higher than that in treated patients ($P = 0.002$). SPAG9 IgG antibody levels were significantly lower in treated adenocarcinoma and small cell lung cancer patients than these levels in the untreated patients ($P = 0.006$, $P = 0.026$, respectively), while no statistical difference was found between treated and untreated squamous cell carcinoma patients. Our results suggest that the SPAG9 antibody in serum is a promising marker for the diagnosis of lung cancer, and the level of the humoral immune response to this antigen appears to be related to the type of lung cancer.

Introduction

Cancer testis antigens (CTAs) are proteins expressed primarily in the testes. These antigens are expressed at very low levels in other normal tissues but have enhanced expression in cancerous tissues (1,2). CTAs do not cause an immune reaction in the testes, but when expressed in cancerous tissue these antigens can induce a specific immune response (3). These characteristics of CTAs make these proteins potential diagnostic markers. Sperm-associated antigen 9 (SPAG9) is a member of the CTA family that is highly expressed in many types of cancers and that causes a strong immune response (4-8). Studies of SPAG9 suggest that it promotes proliferation and invasion (9-11), but whether SPAG9 has clinical value as a therapeutic target or as a diagnostic marker remains to be clarified. At present, there is no early detection test or screening method for lung cancer that accurately and reliably detects the disease in the early stages. In this study, we analyzed expression of SPAG9 in lung cancer tissues and autoantibodies in serum in order to determine the diagnostic value of SPAG9 as a marker of lung cancer.

Materials and methods

Patients and sample collection. Patients were treated at the Tumor Hospital of Hunan Province. Diagnoses were confirmed by pathobiology. Patients consented to specimen collection, and the study was approved by the Ethics Committee of the Second People's Hospital of Hunan Province. Age, gender, tumor size and histological diagnoses of the 20 patients who participated in the tissue arm of the study are shown in Table I. Adjacent non-cancerous tissue specimens were also collected from these patients. It is important to point out that these tissues cannot be regarded as healthy and normal.

Serum samples were obtained from 92 lung cancer patients and 35 healthy subjects. Age and gender of the patients are shown in Table II; there were no significant differences in age or gender among the groups. All cases were diagnosed by CT, MRI, fiber-optic bronchoscopy and percutaneous lung biopsy. Forty-one patients who had not received any treatment were classified as the untreated group. The 51 patients who had received radiotherapy and chemotherapy were classified as the

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Abbreviations: CTA, cancer testis antigen; SPAG9, sperm-associated antigen 9; SCC, squamous cell carcinoma; SCLC, small cell lung cancer.

Key words: lung cancer, SPAG9, autoantibody, biomarker, diagnosis

Table I. Tissue evaluation: Characteristics of the 20 lung cancer patients.

Sample	Age (years)	Gender	Tumor size (cm)	Histological diagnosis
1	55	Male	4.5x3.0x3.0	Moderately and poorly differentiated squamous cell carcinoma
2	45	Male	3.5x2.0x2.0	Moderately and poorly differentiated squamous cell carcinoma
3	70	Male	3.5x3.0x1.5	Moderately differentiated, adenocarcinoma with regional differentiation
4	60	Female	4.5x3.5x3.0	Moderately differentiated squamous cell carcinoma, lymph nodes
5	65	Male	4.0x3.5x3.0	Moderately differentiated squamous cell carcinoma, lymph nodes
6	55	Male	5.0x4.5x2.5	Moderately differentiated squamous cell carcinoma, lymph nodes
7	40	Male	7.5x5.0x4.0	Moderately and poorly differentiated squamous cell carcinoma, lymph nodes
8	52	Male	5.0x3.5x3.5	Moderately differentiated squamous cell carcinoma, lymph nodes
9	66	Male	5.0x4.0x3.0	Moderately differentiated squamous cell carcinoma, lymph nodes
10	55	Male	5.5x4.0x3.5	Moderately differentiated squamous cell carcinoma, lymph nodes
11	51	Female	4.5x3.0x3.0	Moderately and poorly differentiated adenocarcinoma
12	62	Male	5.0x4.0x3.0	Moderately differentiated squamous cell carcinoma, lymph nodes
13	65	Male	5.0x4.5x3.0	Poorly differentiated squamous cell carcinoma, lymph node
14	54	Male	4.5x3.0x2.5	Moderately differentiated squamous cell carcinoma, lymph nodes
15	65	Male	4.0x3.0x1.5	Moderately differentiated squamous cell carcinoma, lymph nodes
16	55	Male	5.5x4.0x2.5	Moderately differentiated squamous cell carcinoma, lymph nodes
17	51	Male	3.5x3.0x2.5	Moderately differentiated squamous cell carcinoma, lymph nodes
18	61	Male	5.0x4.5x4.0	Moderately differentiated squamous cell carcinoma, lymph nodes
19	53	Male	5.0x5.0x4.5	Moderately differentiated squamous cell carcinoma, lymph nodes
20	43	Female	3.0x2.5x2.0	Poorly differentiated adenocarcinoma

Table II. Serum evaluation: Characteristics of the 92 patients and 35 controls.

Characteristic	Untreated group (n=41)	Treated group (n=51)	Healthy controls (n=35)
Age (years)			
Mean \pm SD	60 \pm 11	56 \pm 11	53 \pm 10
Range	36-79	32-75	26-71
Gender			
No. of males	35/41	39/51	26/35
% males	85.4	76.5	74.3

treated group; the characteristics of these patients are shown in Table III. Forty-five of the above 92 lung cancer patients were followed up for 2 years; 25 were deceased and 20 were alive. The age and gender of the patients are shown in Table IV. Peripheral blood was collected, and serum was separated by centrifugation of the blood at 1,000 x g for 10 min. Serum was stored at -80°C until analysis.

RT-PCR testing. mRNA expressed from the SPAG9 gene in tissues was quantified as previously described (12). Total RNA was isolated and cDNA was synthesized using a RevertAid M-MuLV First Strand cDNA Synthesis kit (Thermo Fisher) in accordance with the supplier's protocol. RT-PCR was performed using SPAG9-specific primers. Primers were designed using software Primer 3.0 and were synthesized by

China Yuantai Company. Primers were: homo-SPAG9 forward, 5'-AGCCGACTTTTCAGCTCCTC-3' and reverse, 5'-AAAGCCTGCACTCTACCGTC-3'. Expected fragment length was 114 bp, and the predicted melting temperature was 59°C. The GAPDH mRNA was amplified as an internal control with primers homo-GAPDH forward, 5'-caatgacccttcattgacc-3' and reverse, 5'-gacaagcttcccgtctcag-3'. The expected fragment length was 106 bp. The real-time PCR results were analyzed with SDS 7900 software (ABI).

Western blot analysis. Total protein was extracted from tissue, separated by SDS-PAGE, and transferred to supported nitrocellulose membranes. The protein blots were blocked with 5% milk in PBS overnight at 4°C. Each blot was then incubated with the anti-SPAG9 antibody (PAB8794; Abnova) and mouse GAPDH antibody (sc-166574; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at dilutions of 1:500 in 5% milk prepared in PBS for 2 h at 4°C with gentle shaking. After four 5-10 min washes with PBS-T (PBS with 0.05% Tween-20), each blot was incubated with the secondary antibody (goat anti-rabbit IgG/HRP at a dilution of 1:40,000 or goat anti-mouse IgG/HRP at a dilution of 1:50,000) for 1 h. After four washes with PBS-T, the SPAG9 proteins were detected with an Amersham enhanced chemiluminescence detection kit according to the manufacturer-supplied protocol. After exposure, the X-ray film was analyzed with an ImageQuant LAS-4000 (Fuji). The bands were analyzed using Automated Digitizing System Gel Pro 4.0. The relative expression levels (fold) were calculated by dividing the integrated optical density (IOD) for the band corresponding to SPAG9 by the IOD of the GAPDH band.

Table III. Serum evaluation: Clinicopathology and SPAG9 humoral immune reactions of the untreated and treated patients^a.

Pathological and clinical features	SPAG9 antibody (OD)		Positive/tested (%)	
	Untreated	Treated	Untreated	Treated
All tumors	0.612±0.482 ^b	0.342±0.213	26/41 (63.4) ^b	10/51 (19.6)
Stage of non-small cell lung cancer				
Early (T1 and T2)	0.664±0.642	0.392±0.228	7/13 (53.8)	6/21 (28.6)
Late (T3 and T4)	0.550±0.334	0.365±0.226	10/16 (62.5) ^b	2/17 (11.8)
Indeterminate ^d	0.476±0.254 ^b	0.200±0.09	7/12 (58.3) ^b	2/13 (15.4)
Grade of non-small cell lung cancer				
Low (G1 and G2)	0.542±0.281	0.413±0.188	8/13 (61.5)	3/13 (23.1)
High (G3 and G4)	0.614±0.587	0.378±0.244	9/18 (50.0) ^b	5/24 (20.8)
Indeterminate ^d	0.510±0.276 ^b	0.224±0.127	7/10 (70.0) ^b	2/14 (14.3)
Type of tumor				
Squamous cell carcinoma	0.626±0.540	0.431±0.247 ^c	12/23 (47.1)	8/26 (30.8) ^c
Adenocarcinoma	0.477±0.208 ^b	0.264±0.101	5/7 (71.4) ^b	0/13 (0.0)
Small cell lung cancer	0.844±0.661 ^b	0.235±0.152	4/5 (80.0) ^b	1/8 (12.5)

^aStatistical analysis employed the Fisher's χ^2 method. $P < 0.05$ was taken as a statistically significant association. ^bTransverse comparison, ^cvertical comparison. ^dIncludes small cell lung cancer patient samples.

Table IV. Serum evaluation: Characteristics of the 45 patients who survived 2-years post-diagnosis and those who did not.

Characteristic	Survival group (n=25)	Non-survival group (n=20)	Healthy controls (n=35)
Age (years)			
Mean ± SD	59±11	56±10	53±10
Range	39-75	47-75	26-71
Gender			
No. of males	19/25	16/20	26/35
% males	76.0	80.0	74.3

Immunohistochemistry. Sections (3- μ m) were prepared from the paraffin-embedded tissues. Immunostaining was performed using the two-step EliVision Plus kit (KIT-5020; Maixin). The sections were deparaffinized in xylene, rehydrated with graded alcohol, and then boiled in citrate buffer (pH 6.0) for 2 min in an autoclave. Next, 0.3% hydrogen peroxide was applied to block the endogenous peroxidase activity, and the sections were incubated with normal animal serum to reduce non-specific binding. Tissue sections were incubated with SPAG9 rabbit polyclonal antibody (1:150 dilution; Abcam) for 2 h at room temperature. Rabbit immunoglobulin (at the same concentration as used for the antigen-specific antibody) was used as a negative control. The staining was followed by incubation with polymer secondary antibodies. Color reaction was developed by using 3,3'-diaminobenzidine tetrachloride (DAB) chromogen solution. All slides were counterstained with hematoxylin. Positive control slides were included in every experiment in addition to the internal positive controls.

The specificity of the antibody was determined with matched IgG isotype antibody as a negative control (13).

Sections were evaluated by two investigators in a blinded manner in an effort to provide a consensus on staining patterns by light microscopy (Olympus). Each case was rated according to a score that added a scale of intensity of staining to the area of staining. At least 10 high-power fields were chosen randomly, and >1,000 cells were counted for each section. The intensity of staining was graded on the following scale: 0, no staining; 1+, mild staining; 2+, moderate staining; 3+, intense staining. The area of staining was evaluated as follows: 0, no staining of cells in any microscopic fields; 1+, <30% of tissue stained positive; 2+, between 30 and 60% stained positive; 3+, >60% stained positive. The minimum score when summed (extension + intensity) was, therefore, 0, and the maximum, 6. A combined staining score (extension + intensity) of ≤ 2 was considered to be negative staining (low staining); a score between 3 and 4 was considered to be moderate staining; whereas a score between 5 and 6 was considered to be strong staining. An optimal cut-off level was identified as follows: a staining index score of 0-2 was used to define tumors with negative expression and 3-7 indicated positive expression of these two proteins. Agreement between the two evaluators was 95%, and all scoring discrepancies were resolved through discussion between the two evaluators.

ELISA. Recombinant human SPAG9 protein (r-hSPAG9; Abnova) was used as antigen in an ELISA to detect serum anti-SPAG9 IgG antibody levels (6). Basal levels in the ELISA were established using serum from 35 healthy donors, and the cut-off signal intensity (mean \pm 1.96 SD) was an OD of 0.416 (0.187±0.229). The absorbance was read at 450 with 630 nm as reference filter, and the intra-assay and inter-assay coefficients of variation were 2.3 and 8.6%, respectively.

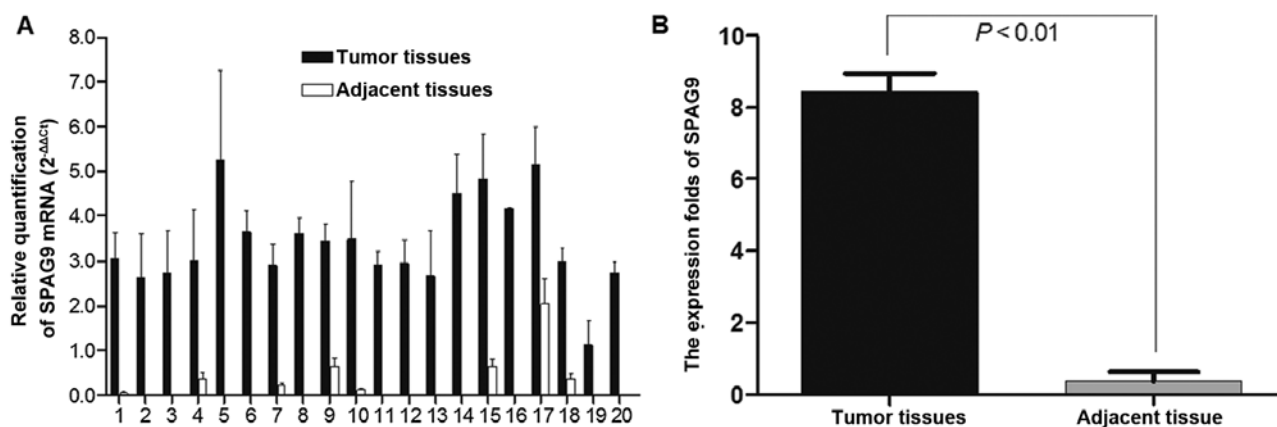


Figure 1. SPAG9 mRNA expression in lung cancer and adjacent non-cancerous tissues was evaluated by RT-PCR with GAPDH as the internal reference. (A) Relative quantification in tumor tissues (black bars) and adjacent tissues (open bars). (B) The normalized SPAG9 gene expression in lung cancer tissues was upregulated by 8.29-fold ($P < 0.01$).

Table V. Tissue evaluation: Immunohistochemistry of the 20 lung cancer and adjacent non-cancerous tissues.

Sample	Grade of staining intensity	Area of staining	Summed score	Result
1	2+	3+	4	Moderate staining
2	1+	1+	1	Negative
3	2+	3+	4	Moderate staining
4	2+	1+	2	Negative
5	3+	2+	4	Moderate staining
6	3+	2+	4	Moderate staining
7	0	0	0	Negative
8	3+	2+	4	Moderate staining
9	3+	2+	4	Moderate staining
10	3+	1+	3	Moderate staining
11	3+	3+	6	Strong staining
12	2+	2+	3	Moderate staining
13	1+	2+	2	Negative
14	2+	3+	5	Strong staining
15	2+	2+	4	Moderate staining
16	3+	3+	6	Strong staining
17	2+	2+	4	Moderate staining
18	3+	2+	5	Strong staining
19	3+	2+	5	Strong staining
20	2+	3+	5	Strong staining

In order to evaluate the response of the immune system to SPAG9, we quantified the serum SPAG9 IgG antibody in peripheral blood of 92 lung cancer patients and 35 healthy subjects; 41 lung cancer patients who had not received any treatment as the untreated group, 51 patients who had received radiotherapy and chemotherapy as the treated group.

Statistical analyses. The Pearson's Chi-square test, Fisher's exact test, Student's t-test for unpaired data, Wilcoxon signed-rank

test, Mann-Whitney U test, and Kruskal-Wallis test were performed using the SPSS 16.0 statistical software. Results are expressed as mean \pm standard deviation (SD). All p-values were two-sided, and a p-value < 0.05 was considered to indicate a statistically significant result.

Results

SPAG9 expression in lung tumors. SPAG9 mRNA levels were quantified by RT-PCR in lung cancer tissues and in adjacent tissues from 20 patients; GAPDH was used as internal reference. The expression of the SPAG9 gene was higher in the lung cancer tissue samples compared with that in the adjacent non-cancerous tissues (Fig. 1A). The normalized SPAG9 gene expression in lung cancer tissues was upregulated by 8.29-fold ($P = 0.000 < 0.01$) (Fig. 1B).

SPAG9 protein expression in the lung tissues was investigated by western blot analysis using anti-SPAG9 antibodies. GAPDH was used as a control (Fig. 2A). SPAG9 expression was significantly higher in the tumor tissues than that in the adjacent non-cancerous tissue (IOD ratio was 0.392 ± 0.104) and was also detected in some adjacent non-cancerous tissues, but with low expression (IOD ratio was 0.03 ± 0.047) ($P < 0.001$) (Fig. 2B and C).

SPAG9 protein expression in lung tissues was investigated by immunohistochemical staining. The samples from the 20 patients were also evaluated by immunohistochemical staining; 20 cases of non-small cell lung cancer tissue (without consideration of the type and stage of cancer) and 20 cases of adjacent non-cancerous tissue specimens were analyzed. Positive staining for SPAG9 was observed in 16 out of the 20 (80%) lung cancer tissue samples. In these cases, SPAG9 protein was localized in the cytoplasmic compartments. No SPAG9 expression was detected in any of the samples of the adjacent non-cancerous tissue (Table V). Representative images of stained tissues from the 20 patients with lung cancer are shown in Fig. 3A and B.

Humoral immune response induced by SPAG9. The serum level of SPAG9 IgG was significantly higher in the 92 lung cancer patients than that in the healthy subjects (0.187 ± 0.117 ,

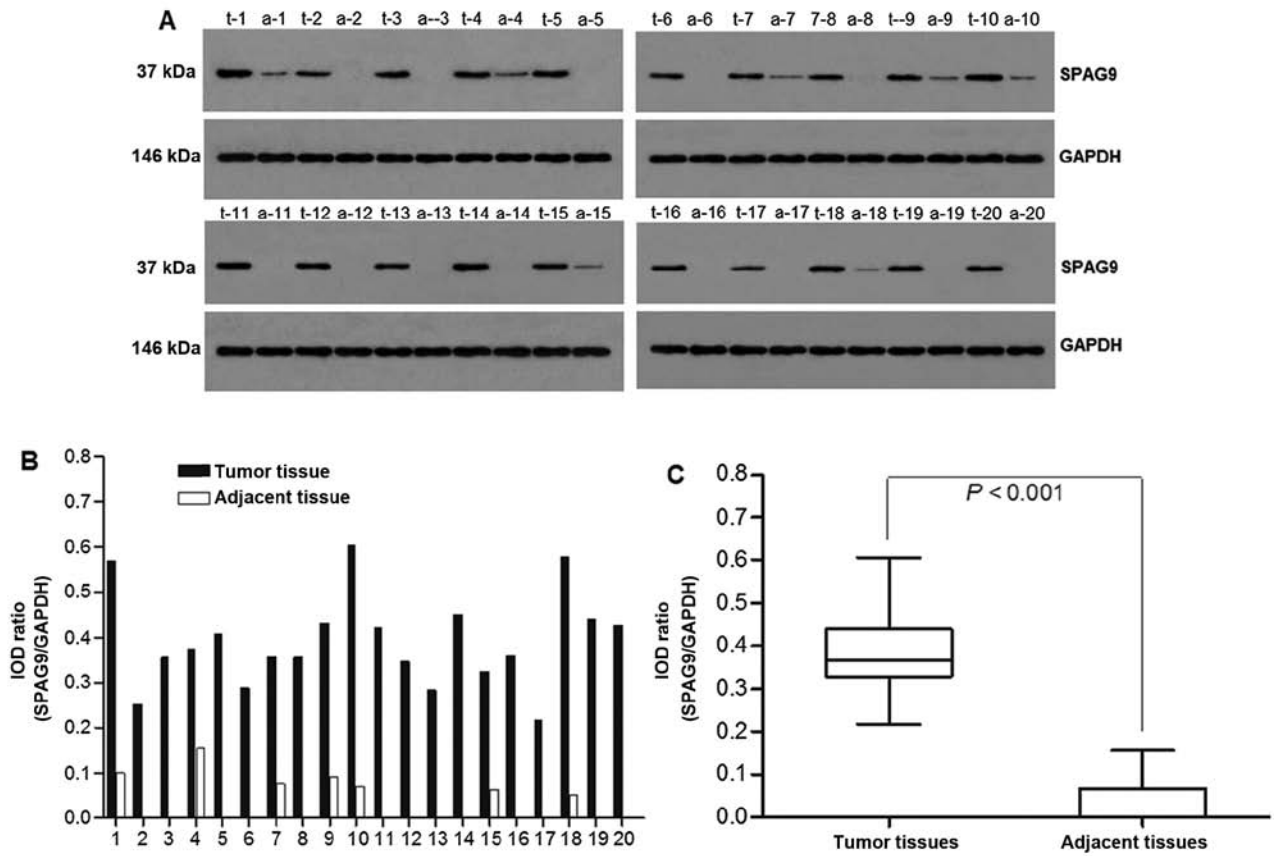


Figure 2. SPAG9 protein levels in lung cancer and adjacent non-cancerous tissues were evaluated by western blotting using GAPDH as internal reference. (A) Western blots of SPAG9 and GAPDH in tumor tissue from representative patients, 't' and 'a' indicate samples from cancerous tissue and adjacent tissues, respectively. (B) Quantification of western blot analysis of tumor tissue (black) and adjacent tissue (open bars). (C) IOC ratio of SPAG9 in tumor tissue was significantly higher than the ratio in adjacent tissue ($P < 0.001$).

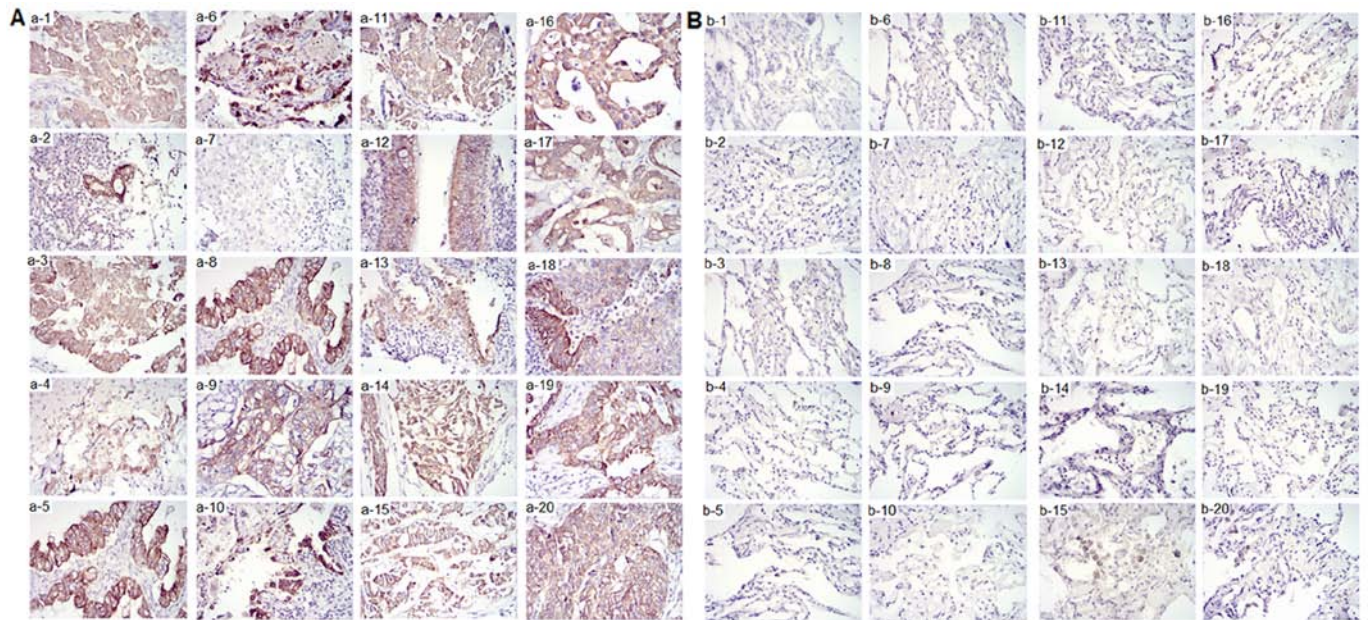


Figure 3. SPAG9 protein expression was analyzed in lung tissues of by immunohistochemical staining. (A) Images of cancerous tissues a1-a20 from patients. (B) Images of adjacent tissue b1-b20 from the same patients. Magnification, x400.

$P < 0.001$) (Fig. 4A). The serum level of SPAG9 IgG was significantly higher in the untreated patients (0.612 ± 0.482)

than that in the treated lung cancer patients (0.342 ± 0.213 ; $P = 0.002$) (Fig. 4B), irrespective of the disease stage. There

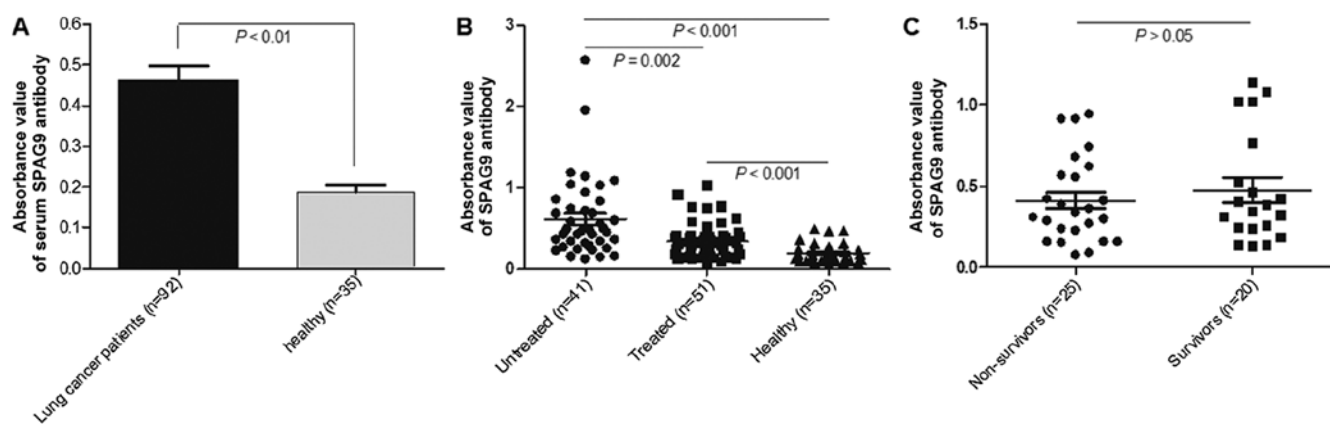


Figure 4. (A) Levels of serum SPAG9 IgG antibody in lung cancer patients and healthy subjects. (B) Levels of serum SPAG9 IgG antibody in untreated patients, treated patients, and healthy subjects. (C) Levels of serum SPAG9 IgG antibody in patients who survived two years post-diagnosis and those who did not. Levels did not differ significantly.

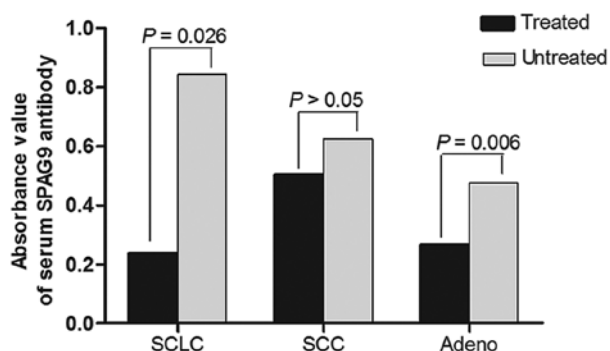


Figure 5. Levels of serum SPAG9 antibody in untreated adenocarcinoma and SCLC patients were significant higher than that in treated group; there was no significant difference in levels of serum SPAG9 antibody between treated and untreated SCC patients. SCLC, small cell lung cancer; SCC, squamous cells carcinoma; Adeno, adenocarcinoma.

were no statistical differences between levels of SPAG9 IgG in the 2-year survivors and the non-survivors (Fig. 4C). No significant differences were observed in serum SPAG9 IgG levels between early stage (T1 and T2) and late stage (T3 and T4) cancer patients or between patients with differentiation grade G1 and G2 and those with low differentiation grades (G3 and G4) irrespective of whether patients had been treated or not. SPAG9 IgG antibody levels were significantly lower in treated adenocarcinoma and small-cell lung cancer (SCLC) patients than levels in the untreated patients ($P = 0.006$, $P = 0.026$, respectively), but no statistical difference was found between treated and untreated squamous cell carcinoma patients (Fig. 5).

Discussion

In China, lung cancer detection is often based on chest X-ray; the disease is usually advanced at the time of diagnosis thus few patients are cured by treatment. If lung cancer is diagnosed early, the chances of a cure can reach 90%; therefore, it is necessary to find methods that enable the early diagnosis of lung cancer.

Some tumor-associated autoantibodies have been detected in patients with lung cancer at the pre-symptomatic stage or before radiographic detection (14-17). CTAs elicit specific humoral immune responses (18) and play an important role in cancer progression (19-22). Therefore, their utility as biomarkers and their potential use in immunotherapeutic strategies are of interest (1,18,23). This study focused on the new CTA SPAG9. Consistent with a previous report (24), overexpression of SPAG9 in lung cancer tissue specimens was detected by immunohistochemistry and SPAG9 protein was localized in the cytoplasmic compartments of tumor cells. The positive rate in this study was higher than that found in the previous study which may be due to the difference in sample size and case selection, or the staining method and the difference in the positive judgment standard. Furthermore, we found that both SPAG9 mRNA and protein expression were higher in lung cancer tissues than levels in the adjacent non-cancerous tissues.

We also discovered that there is a humoral immune response to SPAG9 in lung cancer patients. As a diagnostic marker, serum autoantibodies to SPAG9 were previously shown to be detected in lung cancer patients (25). In our cohort of patients, high expression of SPAG9 IgG antibodies in peripheral blood was observed in newly confirmed lung cancer patients, and low expression in healthy people, indicating that the humoral immune response promoted by SPAG9 was related to the activity of tumor cells. The amount of SPAG9 IgG was lower in the treated lung cancer patients than that in the untreated patients, suggesting that various clinical therapies may reduce SPAG9 expression so as to reduce the humoral immune response caused by SPAG9.

Our study was unable to address whether a decrease in SPAG9 autoantibody levels indicates effectiveness of the treatment. Dynamic observations will be necessary to answer this question. We did find that there was no statistical difference in SPAG9 IgG antibody expression between patients who survived for 2 years after diagnosis and those who did not; therefore, there is no direct relationship between humoral immune response to SPAG9 and patient prognosis. We also found no difference among different disease stages or differentiation grades, and this may be related to the number of

samples or the standard of grading. Thus, we need to expand the sample size and accurate staging in further study.

Adenocarcinoma and SCLC are more likely to spread to the lymphatic and hematogenous systems than SCC, thus easily cause an immune response and produce antibodies. Our results showed that expression of serum SPAG9 antibody in treated adenocarcinoma and SCLC patients was significantly lower than that in the treated SCC patients, but the mechanism is not clear, and the role of the immune response in cancer development warrants further study. However, the marker function of SPAG9 in the course of cancer occurrence and development can undoubtedly help the early diagnosis of cancer.

In summary, the phenomenon of SPAG9 mRNA and protein overexpression in lung cancer tissues was observed, and the SPAG9 IgG antibody was detected in peripheral blood of lung cancer patients indicating that it has potential as a biomarker for lung cancer diagnosis. Whether a decrease in level of the SPAG9 autoantibody correlates with treatment effectiveness requires further study. Our data suggest that there may be differences in the level of expression of the SPAG9 autoantibody in various types of lung cancer and before and after treatment, yet, a larger number of cases need to be evaluated to confirm these results.

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