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Clinically Applicable Diagnostics Assay Development for Lung Cancer Biomarker, SAA

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Abstract

Background: Serum Amyloid A (SAA) is an acute phase protein and has been reported as a lung cancer biomarker. Many cancer protein biomarkers have been reported but few are currently in clinical application. This is due to the obstacles in the process from biomarker discovery to biomarker application: validation in large scale clinical samples and paired antibody production for the development of immunoassay-based diagnostics.

Methods: To develop immunoassay-based diagnostics of SAA, we produced anti-SAA monoclonal antibodies after bacterial production of pure SAA whole protein immunogen. Using two SAA specific monoclonal antibody clones, we developed two types of diagnostics; ELISA and rapid tester diagnostics. Hundreds of clinical samples were tested using the immunoassay diagnostics developed.

Results: The diagnostic or differential diagnostic ability of lung cancer from healthy control or respiratory diseases was tested by developed ELISA or rapid tester kits using hundreds of clinical samples. Developed ELISA kit turned out to measure SAA precisely and clinical sample tests showed that, as previously reported, SAA level is significantly higher in lung cancer groups compared with healthy controls or lung diseases ($p<0.05$). The test results of secondly developed rapid tester kit also showed lung cancer specific positive signals ($p<0.05$).

Conclusion: Both types of immunoassays showed significant diagnostic capability of SAA. This study demonstrated the potential of developed immunoassays for the clinical lung cancer diagnostics.

Keywords: Lung cancer; Biomarker; SAA; Antibodies; ELISA; Diagnostics

Introduction

Lung cancer has been the leading cause of cancer related death worldwide. Compared to other type of cancers, lung cancer still shows the low survival rate. In male and female, cancer showing highest incidence are prostate and breast cancer. However, lung cancer has the highest mortality in both male and female. According to the cancer statistics, 2012, lung cancer shows little increase in 5-year survival rate among all types of cancers along with pancreatic and stomach cancer [1]. This is largely due to the low diagnostic rate of lung cancer in the early stages. At present, diagnosis or prognosis of lung cancer largely depends on imaging tools. Such as X-rays, Magnetic resonance imaging (MRI) and computed tomography (CT) scans are commonly used methods [2].

Although advances in imaging technology have been improved to detect smaller lesions than before, exposure to radiation and the still high proportion of false-positive rate aggravates economic burden and intensify health risks, which signify the support to other type of diagnostic methods for lung cancer In this aspect, protein biomarkers would present new molecular diagnostics, thereby facilitating diagnosis and treatment of lung cancer and its preinvasive state prior to progressive metastasis. Thus, protein biomarkers are expected to be used as pre-screening modalities and auxiliary methods for cancer diagnosis.

Lung cancer is mainly classified into non-small-cell lung cancer (NSLC) and small-cell-lung cancer (SCLC) [3]. NSLC include adenocarcinoma (ADC), squamous lung cancer (SQLC) and large-cell carcinoma, depending on the origin-cell types. Thus far, despite the heterogeneous features of lung cancer, many lung cancer biomarker studies have been carried out. Some known lung cancer biomarkers are cytokeratin-19 fragments (CYFRA 21-1) [4,5], carcinoembryonic antigen (CEA) [6], neuron-specific enolase (NSE) [7], and cancer antigen-125 (CA-125) [8]; however, CEA and CA-125 are pan-tumor markers and are even used for general tumor-marker screenings during physical examinations.

Biomarker application pipeline consists of several steps: discovery, verification, validation, detection probe development, and diagnostic assay development [9]. Discovery phase of protein biomarkers is the step which is most profoundly developed by proteomics technique advances such as mass spectrometry to identify large numbers of proteins, protein labeling for quantitative analysis, and bioinformatics [10]. In a subsequent step, proteins that showed significant changes should be validated and target proteins should be selected [11].

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Biomarkers closely selected from clinical test using thousands samples would be targeted for the development of diagnostics. For this step, detection probe production such as monoclonal antibodies and aptamers to specifically recognize the target protein should precede. After development of assay formats, the diagnostic assays developed should be clinically validated.

Serum amyloid A (SAA) is one of the biomarkers that we have reported as a lung cancer serum biomarker [12]. This protein is known as an acute phase protein which is secreted from liver, which is also highly secreted from the lung cancer cells by inflammatory stimuli. We discovered and validated SAA in the serum of lung cancer patients by ELISA. In our subsequent study, we measured two isotypes of SAA, SAA 1 and 2 by multiple reaction monitoring (MRM) in one hundred serum samples from healthy individuals and another hundred samples from lung cancer patients and validated two proteins as lung cancer serum biomarker [13]. From the two previous studies, we found out significantly higher SAA concentration range in lung cancer patients than in healthy individuals.

In this study, we developed, for the first time, an immune assay based diagnostics by producing SAA specific monoclonal antibodies. Using *E. coli* expression system, we obtained abundant pure SAA proteins to be used as immunogens. By immunization of mice and followed by cell fusion, clonal selection, and ascites production, two SAA specific monoclonal antibodies have been obtained. Using these, two types of diagnostics, ELISA and rapid tester diagnostic kits have been developed. We tested these kits in hundreds of clinical samples to demonstrate the ability of these clinically applicable assays and validate SAA as a lung cancer biomarker.

Materials and Methods

Sample collection

Serum and plasma samples were obtained from the patients at Kyungpook National University Hospital (IRB #; KNUMCBIO_11-0001). Informed consent was obtained from all donors. The samples were separated from whole blood by centrifugation within 4 h after collection and stored at -70°C until use. The patient information for both sample groups is summarized in Tables 1 and 2.

Cloning and preparation of expression vector

Full-length SAA was cloned into pPAL7, *E. coli* expression vector (Bio-Rad, USA). Detailed information is described in results and supplementary information.

Sample types		Sample No. Tested (Test 1/Test 2)	Sex(M/F)	Age
Healthy		82/0	42/40	59.5 (45-86)
Lung Diseases		0/177	96/81	55.9 (21-84)
Lung cancer	AdenoCA	69/119	90/98	60.8 (32-83)
	SQLC	61/58	109/10	65.4 (44-83)
	SCLC	0/26	26/0	65.9 (52-82)
	Large cell CA	4/0	2/2	57.0 (50-60)
	Others	23/10	27/6	63.7(51-78)
	Total	157/213	253/117	62.9 (32-83)

*Test 1 indicates ELISA test of lung cancer vs healthy control (Figure 3B~E). Test 2 means comparison analysis between lung cancer and lung diseases (Figure 3F~H).
**Ages are given as average (range).
***Abbreviations used: AdenoCA; adenocarcinoma, SQLC; squamous cell carcinoma, SCLC; small cell lung cancer

Table 1: Information of clinical samples tested for ELISA.

Sample types		Sample No. Tested	Sex (M/F)	Age
Healthy control		150	85/65	64.8 (21-85)
Lung cancer	AdenoCA	150	102/48	66.1 (32-79)
	SQLC	50	44/6	63.3 (49-79)
	SCLC	48	46/2	63.1 (42-83)
	Total	248	192/56	64.0 (32-83)

**Ages are given as average (range).
***Abbreviations used: AdenoCA; adenocarcinoma, SQLC; squamous cell carcinoma, SCLC; small cell lung cancer

Table 2: Clinical sample Information of SAA Rapid kit.

Protein expression and purification

The protein expression and purification of expressed SAA proteins were carried out following the manufacturer's instructions. Detailed information is also stated in supplementary information.

Monoclonal antibody production

Traditional monoclonal antibody production procedures, mouse immunization, hybridoma cell fusion and ascites production were carried out. The whole procedure was described in results and supplementary information.

ELISA

Samples were analyzed after 75-fold dilution. One type of monoclonal antibody to SAA were used for capturing and another HRP conjugated-monoclonal antibody to SAA were used for detection. Development was performed using tetramethylbenzidine (TMB) substrate. The optical density was read on an ELISA plate reader (Epoch, BioTek Instruments, Inc.)

Western blot analysis

Western blot analysis was performed as previously reported [12]. Briefly, 100 ng of SAA proteins were separated by SDS-PAGE then transferred to PVDF membrane (Millipore Corporation). The membrane was incubated overnight with the SAA antibody or the supernant of hybridoma followed by horseradish peroxidase-conjugated anti-mouse IgG secondary antibodies (Enzo).

Statistical analysis

For statistical analysis, the group results were subjected to statistical analysis with non-parametric Kruskal-Wallis test to assess differences between groups. A probability (P value) less than 0.05 was considered to be statistically significant. The area under the curve (AUC) value was determined using the OriginPro 8.

Results

Preparation of pure SAA protein as an immunogen

For the immunization to the mouse, large amount (about 3~5 mg) of protein immunogen is required. To obtain SAA pure protein, SAA coding sequence cDNA was cloned into pPAL7 *E. coli* expression vector which contains Profinity eXtag sequences right before the multiple cloning sites (MCS) (Figure 1A). It appears that after restriction enzyme cut of cloned plasmid, pPAL7 vectors and SAA gene appears at the length of 5.9 kbp and 300 bp each (Figure 1B). IPTG (Isopropyl β-D-1-thiogalactopyranoside) induction of *BL21 E. coli* competent cells transformed with SAA gene resulted in high expression of eXact tag (8 kDa) fused-SAA protein in the soluble parts of cell lysates. The SAA proteins were purified and the purity and exact size were confirmed by SDS-PAGE and Coomassie brilliant blue (CBB) staining (Figure

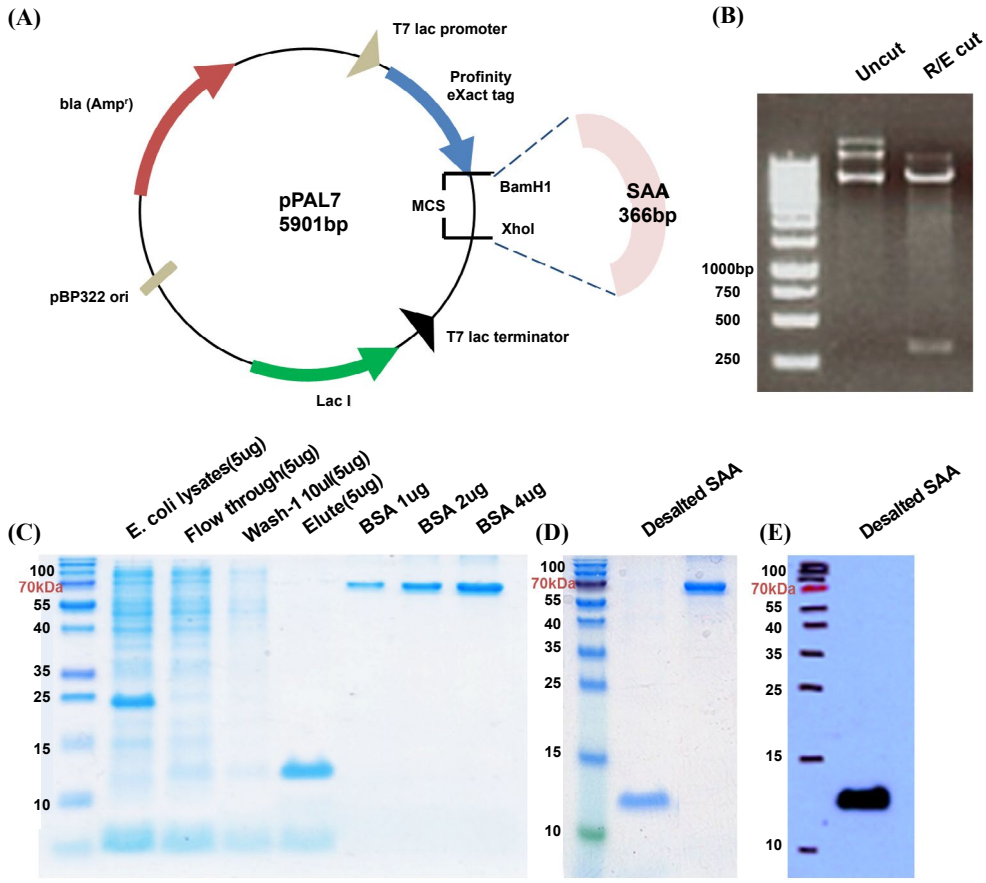

Figure 1: Production of pure synthetic SAA protein by *E.coli* expression systems
(A) Full length ORF of SAA was cloned into *pPAL7* vector by restriction enzymatic digestion. **(B)** Cloned vector was confirmed by electrophoresis of plasmid cut with two restriction enzymes, BamHI and XhoI, used for the cloning. **(C)** Commassie Brilliant blue staining of SDS-PAGE shows the results of whole procedures of protein expression and purification process. IPTG treated E.coli lysates showed the major band at the molecular weight of 20 kDa which contains highly expressed SAA (12 k Da) with Profanity eXact tag (8 kDa). Pure SAA without tagging protein was obtained after immunoaffinity purification. SAA proteins were not present in flow through or wash contents during the process. **(D)** Elutes containing the proteins with low concentration were enriched by 3 kDa-cut off filtrations to 1.5 mg/ml concentration. The concentrated proteins were confirmed by Bradford assay and SDA-PAGE visualized by Coomassie Brilliant Blue (CBB) staining. **(E)** Desalted SAA was also confirmed by Western blot analysis with commercial SAA polyclonal antibody.

1C). After the removal of the salts in the elution by 3 kDa cut-off filter, the concentration of desalted SAA protein was measured by Bradford assays and by comparing to BSA standard in CBB staining after SDS-PAGE (Figure 1D). Finally the SAA protein was further confirmed by western blot analysis with commercial SAA antibody (Figure 1E).

Production of anti-SAA mouse monoclonal antibodies

To develop immunoassay based diagnostics of SAA, production of SAA-specific monoclonal antibodies is the first step (Figure 2A). Mice were immunized with purified SAA proteins. The increase of antibody titer in the mice was confirmed after the 2nd and the 3rd immunization (Figure 2B). Spleen cells of the immunized mice were fused with murine multiple myeloma cell lines, SP2/0. Among the fused hybridoma cells, single cell clones showing antibody secreting activity were selected and immunoreactivity was measured by direct ELISA and Western blot analysis. An example of western blot analysis showed 10G1 as a good clone candidate compared to other clones selected from first mouse (Figure 2C). The clones showing good immunoreactivity were injected into mouse abdominal cavities to induce ascites production to acquire large quantity of antibodies. Through IgG affinity purification, antibodies were purified from ascites (Figure 2D). Eluted fraction was combined and immunoreactivity of produced antibodies was tested on immunogens by ELISA and western blot analysis (Figure 2E).

Development of SAA ELISA diagnostics and clinical tests

Utilizing the produced anti-SAA monoclonal antibodies, we developed two different types of diagnostic assay platforms. First, sandwich ELISA type of diagnostics was produced. From the several clones of hybridomas, one combination of two clones showing best standard curve in sandwich ELISA format was selected.

To confirm the purity of immunogen made from *E. coli* and sensitivity and specificity of developed ELISA, SAA purchased from the National Institute for Biological Standards Control (NIBSC) was used and the result was compared with standard curve made from SAA protein used as immunogen (Figure 3A). In the results, both standard curves appeared with good linearity and the measured OD values were similar. Also in the clinical sample tests, the SAA concentration calculated from two different standard curves were almost same (Supplementary Table 1).

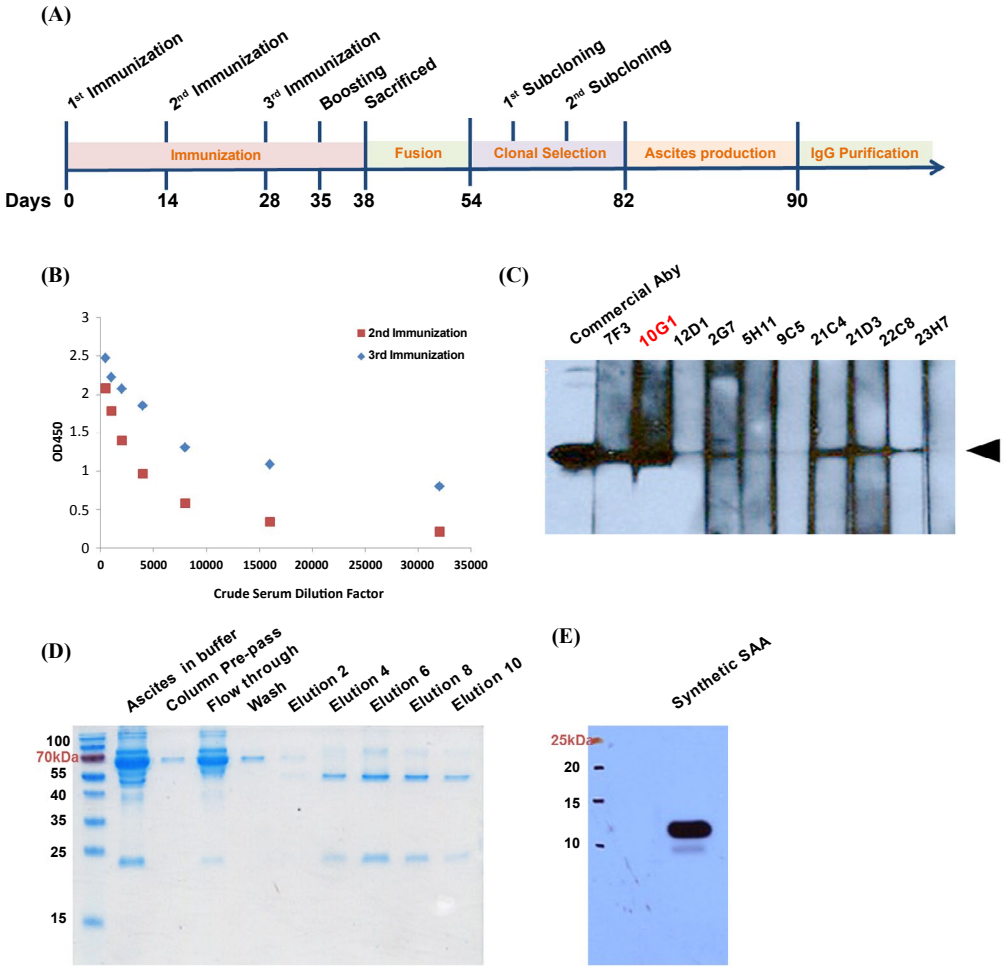

Figure 2: Mouse immunization and production of SAA monoclonal antibodies
(A) Monoclonal-antibody production consists of sequential procedures: immunogen production, immunization and boosting, cell fusion, clonal selection, ascites production and antibody purification; overall, monoclonal antibody production takes approximately 7 months. **(B)** Antibody titer in the blood and the level of immunization was regularly assessed by direct ELISA of crude serum on immunogen coated plates. After the second and third immunization, 10 ul of blood of immunized mice was tested. **(C)** Supernatant of each clone was used for the western blot analysis to detect SAA protein, used in the immunization. Clones were selected by the results of western blot analysis. **(D)** Pure antibodies were purified from ascites by passage through IgG immunoaffinity columns. The CBB staining results show that highly purified IgG against hu-SAA. **(E)** Purified anti-SAA antibody sensitivity and specificity was tested by western blot analysis on the synthetic SAA.

Using SAA sandwich ELISA developed, two large scale validations was carried out. First, lung cancer diagnostic ability of SAA compared to healthy individuals was tested. Serum of 82 healthy individuals and 157 lung cancer patients were subjected for the experiment (Table 1 and Supplementary Table 2). The result showed significant difference between lung cancer groups and healthy individual group; the average SAA concentration measured in healthy individuals was 2.684 μg/ml and that of lung cancer group was 6.147 μg/ml (Figure 3B). The results were also statistically analyzed by receiver operating characteristic (ROC) curves. The sensitivity and specificity was 0.70 and 0.74 respectively and Area under curve (AUC) was 0.741 (Figure 3C). Sera of 157 lung cancer patients include those of 69 lung adenocarcinoma patients (AdenoCA) and 61 squamous lung cancer (SQLC) patients. Statistical significance of difference between healthy individuals and each histological types of lung cancer was also analyzed. Both group showed significant difference compared to healthy individual group and p-value was much lower in comparison of SQLC group with healthy group (Supplementary Table 3). In the analysis of ROC curves, AUC of AdenoCA group and SQLC group showed 0.694 and 0.761 each in comparison with healthy group (Figure 3D and 3E). The diagnostic capability, sensitivity, of SAA for AdenoCA and SQLC was 0.61 and 0.74 with same specificity of 0.74.

Second, differential lung cancer diagnostic ability of SAA from lung diseases was tested. Serum of 213 lung cancer patients and serum of 177 individuals with various lung diseases was subjected to the ELISA analysis. Various lung diseases include common lung diseases such as, tuberculosis, pneumonia, bronchiectasis, atelectasis and asthma etc. The average SAA concentration of lung diseases group and lung cancer group were 1.510 μg/ml and 3.652 μg/ml respectively and showed significant difference (Figure 3F, 3G and Supplementary Table 4). Among various lung diseases, tuberculosis is well known for its differential diagnostic difficulty from lung cancer in the result of chest X-ray carried out for screening of lung cancer. Results of 33 patients with tuberculosis were selected and compared with lung cancer. Tuberculosis patient group showed greater difference compared to lung cancer than combined lung diseases group in SAA concentration level.

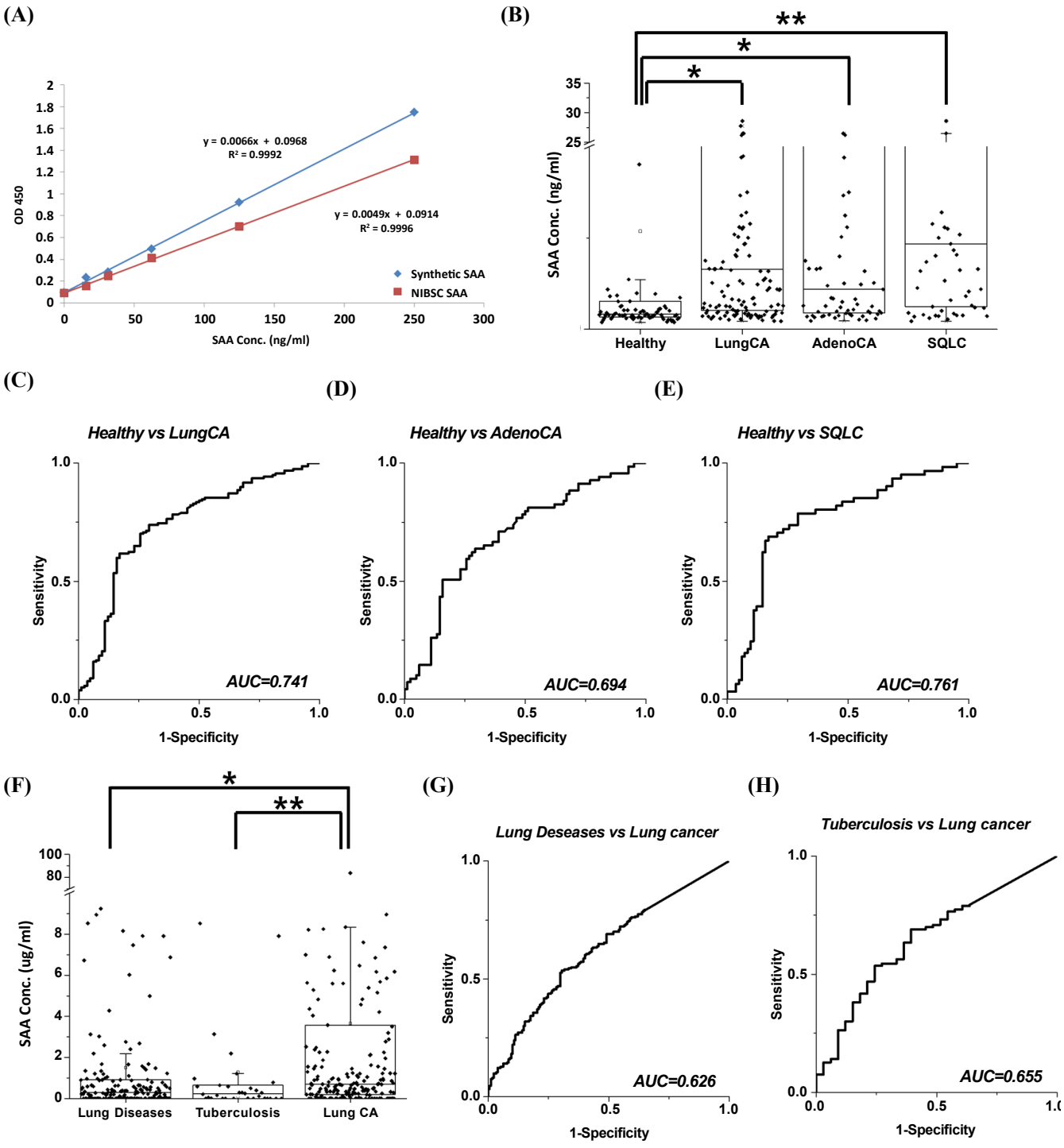

Figure 3: Development of ELISA and clinical sample test

(A) With the SAA monoclonal antibodies produced, ELISA system was developed. Standard curve on synthetic SAA and NIBSC standard SAA shows good activity of the SAA ELISA developed. **(B)** Total 239 clinical samples were tested with the ELISA; 82 healthy individuals and 157 lung cancer patients. A significant difference was observed between healthy individuals and lung cancer or healthy individuals and two lung cancer histological types; adenocarcinoma, or squamous lung cancer. **(C, D, E)** The Diagnostic ability was evaluated by ROC curve and its AUC value. **(F)** One hundred seventy seven sera from lung disease patients and 213 sera from lung cancer patients were subjected to ELISA test to verify the ability of the SAA to differentiate between lung cancer and other lung diseases. A significant difference was observed between lung diseases and lung cancer. Differential diagnostic ability from tuberculosis was evaluated and significant difference was confirmed. **(G, H)** The overall differential diagnostic ability is shown in an ROC curve. (* indicates P <0.05, ** denotes p<0.001 compared to each groups in analysis of significant variance).

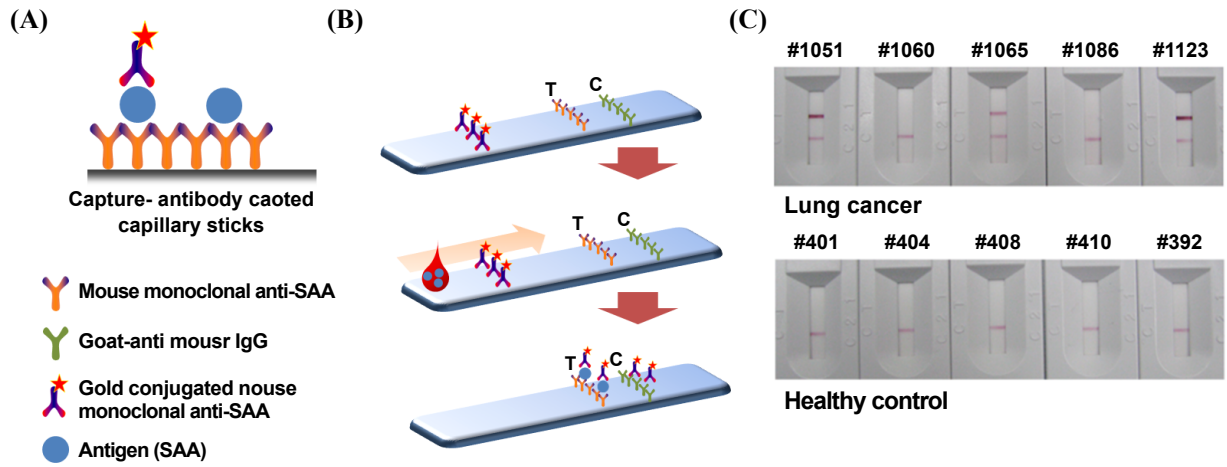

Figure 4: Development of rapid strip diagnostics kit and clinical sample test
(A) Schematic draw of the principles of rapid strip kit is shown. **(B)** Experimental flow is summarized in graphical diagram. **(C)** Pictures of representative test results are shown. Most of healthy individuals were negative, showing only one control line. More than half of the lung cancer patients showed two lines, one for the control and another for the elevated SAA. The test results are summarized in Table 3.

Sample types		Sample No. Tested	Samples No.		Sensitivity	Specificity
			Negative	Positive		
Healthy control		150	146	4		97.33
Lung cancer	AdenoCA	150	72	78	52.00	
	SQLC	50	15	35	70.00	
	SCLC	48	21	27	56.25	
	Total	248	108	140	56.45	

Table 3: Clinical sample test result of SAA Rapid kit.

The AUC value (0.655) was also higher than combined lung diseases group (Figure 3H). ELISA results of 50 patients with bronchial asthma, another one of the most common respiratory diseases, were also compared with lung disease group. SAA concentration of bronchial asthma group was 1.758 μg/ml, significantly lower to the lung cancer group, 3.652 μg/ml (Supplementary Table 4).

Development of SAA rapid strip diagnostics and clinical tests

As a second immunoassay type, SAA rapid strip diagnostic kit was developed. Same two monoclonal antibody combination used in ELISA was used. The difference in detection was that for rapid strip diagnostics, detection antibody was conjugated with gold particles instead of HRP enzymes (Figure 4A). SAA capture antibody was coated on the nitrocellulose membrane and with some distance, capture antibody for goat anti-mouse IgG was coated on the control line which indicates normal flow of sample to the end of membrane. The usage procedures for the rapid diagnostic kit are simple. First, after applying 10 μl of serum on the sample hole, two drops of capillary buffer is applied to assist the flow of sample through the paper and wait for 10 min for the reaction. If SAA exists in the serum above the concentration of detection criteria, two lines will appear, otherwise, only one control line will appear (Figure 4B).

We tested this kit with clinical samples of 150 healthy individuals and 248 lung cancer patients including adenocarcinoma, squamous lung cancer, and small cell lung cancer. Only 4 individuals from healthy control group showed positive correlation, however, 140 out of 248 individuals of lung cancer patients group showed positive results in the test. This means the SAA rapid strip kit showed 56.45% sensitivity and 97.33% specificity in overall lung cancer types. The SQLC showed especially high sensitivity of 70% (35 positive out of 50 samples) (Figure 4C and Table 3). When the results were analyzed by cancer stages, there was no significant difference, indicating that SAA could diagnose lung cancers in regardless of the lung cancer stages (Supplementary Table 5).

Discussion

In this study, we developed clinical application diagnostic kits for SAA, previously reported and studied as a lung cancer diagnostic biomarker by our own group and tested the kits on hundreds clinical samples. Thus, we showed a whole new pipeline from the discovery of biomarkers, production of specific paired monoclonal antibodies, and development of immunoassay based diagnostics, to the clinical test using the kits.

For a clinically applicable assay for protein biomarkers, antibodies, the most specific and sensitive probes for proteins, are essential for both diagnostics and therapeutics [14]. The most important factors for antibody production are high specificity and sensitivity with possible continuous production of homogeneous antibodies. For this reason, monoclonal antibodies are the best type of antibody for the immunoassay development because monoclonal antibodies secrete an antibody for single epitope and can be produced with same quality from hybridoma cells continuously [15].

Antibody-based immunoassays are the most appropriate methods for the detection and measurement of protein biomarkers. Among various types of immunoassay-based diagnostics, Enzyme-linked immunosorbent assay (ELISA) is the most broadly used protein-quantification method. ELISA can measure protein concentrations in hundreds of samples at one time. In addition, sandwich ELISA is high sensitive method among most widely used antibody-based assay and can detect ten to hundreds of nanograms per milliliter. As most broadly used immunoassay, ELISA is used not only in research but also in clinics. Several tumor markers, such as CEA, AFP and CA-125,

are measured by double-antibody sandwich ELISA or a modified form such as CLIA (Chemiluminescence Immunoassay) during medical examinations in many hospitals [16]. Development of sandwich ELISA for lung cancer biomarkers will give more accurate and sensitive information with exact protein concentrations.

Secondly developed type of diagnostic method is called rapid-test kit, rapid strip kit, because the results are available within 5 to 10 min. The best-known rapid diagnostic test kits are pregnancy test kits, which measure highly elevated human chorionic gonadotropin (hCG) levels in the urine of pregnant women [17]. For lung cancer diagnosis, no rapid diagnostic test kit has been developed. This type of assay format does not require a laboratory or any other special equipment; therefore, rapid kits for cancer diagnosis might be widely used in screening and as a supportive diagnostic tool.

In this study, we presented an example of clinical application of serum protein biomarker discovered and validated by proteomics. In the results, additional clinical test using developed immunoassays has confirmed the potency of SAA as a lung cancer diagnostic biomarker. Furthermore, the differential diagnostic capability has been also suggested in this study. However, the characteristics of two different types of diagnostics suggested different usage of these two methods in clinics. Although ELISA needs to be conducted in the laboratory and takes several hours, it can give exact SAA concentration and the value could provide more information than rapid kit. On the other hand, rapid diagnostic kit could be tested in 10 minutes and conveniently tested by users. However, the false positive could give great confusion to the testers, the diagnostic range would be set higher and this will lead to low sensitivity and false negatives. Both diagnostics cannot be ideal diagnostic methods for lung cancer, however they might provide screening and supportive modelaties for the current lung cancer diagnosis.

Moreover, from the experience of composing SAA Rapid tester kit and ELISA kit, we learned that the sensitivity and specificity of the monoclonal antibody are the key to the successful development of diagnostics. Also, deciding the detection criteria and optimizing the detection ranges for the biomarker detection are also important for the production of good diagnostic kits. In addition, it would be another challenging task for the researchers of protein biomarkers to lead the novel biomarker findings to clinics. With the ultimate development of this novel standardized assay, it is expected that suitable protein biomarkers would be validated aided by the integration of proteomic technology thus having a utility for the early detection of lung cancer within the next few years.

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Disclosure

A coauthor, Dr. Kyung-Jo Seul, is an employee of ProtAnBio Co and her salary comes from ProtAnBio Co.

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