Evaluation of kininogen 1, osteopontin and α-1-antitrypsin in plasma, bronchoalveolar lavage fluid and urine for lung squamous cell carcinoma diagnosis

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Abstract. Lung squamous cell carcinoma (LUSC) progression is accompanied by changes in protein levels that may be reflected in body fluids, such as plasma, bronchoalveolar lavage fluid (BALF) and urine. Certain proteins present in these biofluids can facilitate lung cancer diagnosis. Kininogen 1 (KNG1), osteopontin (OPN) and α-1-antitrypsin (AAT) are associated with tumorigenesis. The present study aimed to explore the combined monitoring of plasma, urine and BALF to gain insight into LUSC by monitoring the levels of the above three protein using ELISA. LUSC (n=31) and healthy controls with benign lung diseases (n=20) were enrolled in the study. KNG1 levels in plasma, BALF and urine were significantly higher in patients with LUSC patients than in controls (P<0.0001, P<0.0001 and P=0.0010, respectively). OPN was upregulated in the plasma and BALF of patients with LUSC relative to controls (P=0.0107 and P=0.0004, respectively), whereas its levels in the urine of healthy controls were significantly higher (P=0.0088). Patients with LUSC had higher AAT levels in plasma, BALF and urine compared with those of the controls (P=0.0022, P=0.0014 and P=0.0005, respectively). Receiver operating characteristic analysis showed an area under the curve (AUC) of 0.81 for KNG1 in plasma, 0.91 in BALF and 0.81 in urine. The AUC for OPN was 0.71 in plasma, 0.83 in BALF and 0.75 in urine. The AUC for AAT was 0.74 in plasma, 0.74 in BALF and 0.86 in urine. Immunohistochemical staining in 20 paired LUSC and adjacent normal tissues showed that KNG1, OPN and AAT levels were higher in LUSC tissues. Therefore, our results showed that KNG1, OPN and AAT in biofluids might be useful for the diagnosis of LUSC. These markers in urine and BALF may be better than in plasma for detecting LUSC.

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Introduction

Lung cancer is the leading cause of cancer death in the world, accounting for >1/4 of all cancer-related deaths (1). Almost 85% of patients with lung carcinoma exhibit non-small cell lung cancer (NSCLC), of which lung squamous cell carcinoma (LUSC) accounts for ~30% and results in ~400,000 deaths annually (2). The primary strategy for LUSC treatment at present remains surgical resection. However, this treatment is generally not effective once the disease progresses to a metastatic stage. Individuals with advanced disease have a poor prognosis. Indeed, chemotherapy generally fails to treat patients with metastatic LUSC, and this disease has a <20% 5-year survival rate, with no optimal targeted therapeutic having yet been identified to treat this disease (1). The low survival rate of patients with LUSC is at least partially attributable to the disease often not being diagnosed until it is relatively advanced, thus precluding surgical treatment (2). The present study aims to provide a useful reference in the future diagnosis of LUSC.

Cancer cells and normal cells exhibit distinct patterns of protein production and secretion, with numerous tumors exhibiting marked shifts in proteolytic activity as their signaling alters during the progression towards malignant disease (3). As such, it is possible to detect specific cancer-associated proteins in the biofluids of patients, and these proteins as biomarkers can offer an insight into disease type and stage. Such biomarkers have been sought as a means of facilitating LUSC diagnosis and monitoring, since their detection is easier than a more invasive biopsy procedure and allow rapid screening. Minimally invasive tumor biomarkers that are readily accessible in biofluids such as plasma, urine and bronchoalveolar lavage fluid (BALF) would thus offer a means of easily and effectively differentiating between patients with cancer and those with benign disease (4-6). Urine markers can be detected without exposing individuals to any risk, and urine is highly amenable to large-scale screening efforts. Therefore, urine is a particularly promising biospecimen for biomarker screening.

In theory, such an approach would allow population-level screening of individuals, thereby facilitating the early detection of LUSC and other types of cancer. However, the current biomarkers for the diagnosis of LUSC are mainly blood tumor markers such as squamous cell carcinoma (SCC) antigen and cytokeratin 19 fragment 21-1, but their sensitivity and specificity are low. There arw few studies on biomarkers of LUSC in easily accessible specimens, such as urine and BALF (4,5).

Kininogen 1 (KNG1) is a cysteine proteinase inhibitor known to inhibit endothelial cell proliferation and angiogenesis (7). Certain groups have reported the detection of KNG1 using mass spectrometry, and recently KNG1 has been identified as a serum biomarker for advanced colorectal adenoma and colorectal cancer, in addition to being a salivary biomarker useful for oral SCC detection and for the monitoring of high-risk individuals (8,9). However, the disease relevance of KNG1 levels in patients with LUSC remains to be assessed. Osteopontin (OPN) is expressed in a variety of tissues, and is abundant in body fluids such as blood, milk and urine (10). It is a multifunctional phosphorylated glycoprotein involved in cell migration and adhesion, and mediates the invasion and metastasis of tumor cells (6). OPN is associated with tumorigenesis, progression, metastasis and cancer prognosis (11). α-1-Antitrypsin (AAT) is also known as serine proteinase inhibitor A1. It was reported that the level of AAT was elevated in the tissues and serum of patients with lung cancer, wherein it was thought to promote tumor invasion and metastasis (12-14). At present, the levels of OPN and AAT in the urine and BALF of patients with lung cancer remain unclear. Moreover, the combined assessment of the diagnostic relevance of KNG1, OPN and AAT levels in urine, BALF and plasma have not been studied in LUSC thus far.

The present study aimed to assess whether KNG1, OPN, and AAT levels in specific biofluids, as measured by ELISA, may be a viable diagnostic biomarker for LUSC. In addition, the levels of these three proteins in LUSC tissues were assessed via immunohistochemistry (IHC).

Materials and methods

Study subjects. Patients with LUSC patients and controls were recruited from Beijing Shijitan Hospital between October 2014 and March 2017. The Ethics Committee of Beijing Shijitan Hospital, Capital Medical University approved the present study (approval no. 10, 2014). All study participants provided written informed consent, and the study was performed according to the Declaration of Helsinki. For patients with LUSC, two senior pathologists confirmed the diagnosis based on pathology findings. The control group included various benign lung disorders such as chronic cough, benign pulmonary nodules, hemoptysis, bronchitis, sarcoidosis, asthma, bronchiectasis and tuberculosis. The characteristics of the patients with LUSC and the control subjects are presented in Table I. All plasma, BALF and urine samples were collected prior to radiological, surgical or chemotherapeutic treatment. There was no evidence of hematuresis in any urine samples, with all albumin/creatinine ratios in urine samples being <30 mg/g. From 20 of the total number of patients in this study, pairs of LUSC tumor tissue and adjacent normal tissue located ≥5 cm from the tumor site were also used. Patients who underwent preoperative radio- or chemo-therapeutic treatment were excluded from the study. Patients with LUSC were classified based on the 2009 TNM classification system for malignant tumor staging produced by the International Union Against Cancer and the American Joint Committee on Cancer.

Plasma collection. From each subject, 6 ml of venous blood was collected using closed syringes containing a coagulation activator, and samples were then immediately centrifuged at 3,600 x g for 10 min. The plasma fraction was transferred to a separate Eppendorf tube and stored at -80˚C. Of note, subjects were fasting at the time of sample collection.

Urine collection. A total of 50 ml of mid-stream urine from each subject was collected into a sterile polypropylene tube. Samples were immediately centrifuged at 400 x g for 15 min, and the supernatant was then aliquoted and frozen at -80˚C.

BALF collection. Patients were first administered 2% lidocaine for local anesthesia. Subsequently, a fiber-optic bronchoscope (Olympus EXERA BF 240; Olympus Corporation) was used to perform a bronchoscopy. Lavage was conducted before biopsy or brushing-based specimen collection to prevent any possibility of blood contamination. Lavage was performed by washing the bronchus of the side affected by the disease twice with 50 ml sterile saline solution, and then slowly withdrawing this solution into a siliconized tube and placing it in ice water. A recovered BALF volume of 40 ml was considered acceptable. After isolation, debris and cells were immediately removed via centrifugation at 1,500 x g for 10 min, and the supernatants were subsequently frozen at -80˚C.

ELISA. The KNG1, OPN and AAT levels in plasma, urine and BALF were measured using a commercially available ELISA kit Abnova, Abcam and Abcam, respectively, according to the manufacturer's instructions. Plasma and BALF samples were diluted 200- and 100-fold, respectively. Next, plasma, BALF and urine samples were incubated in KNG1 ELISA plates. Urine samples were diluted 1,500-fold and then plasma, BALF and urine samples were incubated in OPN ELISA plates. The AAT ELISA plates were incubated with plasma, urine and BALF samples at dilutions of 1:400,000, 1:400 and 1:500, respectively. The optical density was measured at 450 nm with a Model 680 microplate reader (Bio-Rad Laboratories, Inc.). A standard curve was drawn for each plate using the concentration of the standard sample and the corresponding optical density value of each well. Both positive and negative controls were used for validation.

IHC. Following surgical collection, LUSC and adjacent healthy tissue samples were formalin-fixed, paraffin-embedded and cut into 4-mm sections. Next, xylene was used to deparaffinize the samples for 20 min, and an ethanol gradient (100, 100, 95 and 75%, 2 min each) was then used to dehydrate samples. PBS was next used to wash the samples (5 times, 10 min each), and the samples were then heated under pressure with an antigen unmasking reagent to facilitate antigen retrieval. After an additional 10-min wash with PBS, 3% H_2O_2 was used to treat the samples for 15 min, followed by an additional wash

Table I. Demographics of patients with cancer and control subjects.

Characteristics	Biofluids set		Tissue set	
	Patients with cancer (n=31)	Normal controls (n=20)	P-value	Tumor/adjacent normal pairs (n=20)
Age, years	65.7±9.7	67.4±9.4	0.55	63.0±8.2
Sex				
Female	10 (32%)	8 (40%)	0.57	6 (30%)
Male	21 (68%)	12 (60%)		14 (70%)
Smoking habit				
Nonsmoker	11 (35%)	10 (50%)	0.30	8 (40%)
Ever smoker	20 (65%)	10 (50%)		12 (60%)
Clinical stage				
I-II	13			9
III-IV	18			11
Pleural invasion				
Absent	27			20
Present	4			0
Lymphatic invasion				
Positive	25			15
Negative	6			5

step. Tissues were then probed overnight at 4˚C with appropriate primary antibodies against OPN (1:300, Abcam), AAT (1:300, Abcam) and KNG1 (1:200, Abnova). Samples were again washed and then probed for 20 min with a secondary antibody conjugated to HRP (Beijing Zhongshan Jinqiao Biotechnology, Co., Ltd.) at 37˚C the following day. An additional 15-min wash was then performed, and the chromogenic 3,3'-diaminobenzidine mixture(Beijing Zhongshan Jinqiao Biotechnology, Co. Ltd.) was next used to stain the samples for 5 min. Hematoxylin was used for counterstaining for 2 min, and then the samples were dehydrated with ethanol (75, 95, 100 and 100%) and washed with xylene, and natural gum was used to seal the samples.

Two independent pathologists blinded to the patients' information independently assessed the IHC slides via light microscopy. A Nikon Ci-S (Nikon Corporation) microscope with NIS-Elements F software (Nikon Corporation) was used to capture images of the samples. Sample scoring was conducted as in previous studies based on 10 different fields of view (15). Both staining intensity (intensity) and area (extent) were scored for each sample. With respect to intensity, samples were scored as either 0, 1, 2, or 3, which corresponded to no, mild, moderate, or intense staining, respectively. With respect to area, samples were scored as either 0, 1, 2, 3, or 4, which corresponded to 0, 1-10, 11-50, 51-80, and 81-100% of positive cells, respectively. These two scores were multiplied together to yield an overall score, with overall scores of 4-12 being considered positive, and scores of 0-3 being considered negative.

Statistical analysis. All statistical analyses were conducted using SPSS v22.0 (IBM Corp.). Normally distributed data were compared via Student's t-tests, while Mann-Whitney U tests was used for comparisons of non-normally distributed data. The sensitivity and specificity of these biomarkers were assessed based on the area under the curve (AUC) of the receiver operating characteristic (ROC) curve. χ^2 test was used to assess the baseline characteristic differences between the LUSC and control groups, and to compare the proteins levels in LUSC and adjacent normal lung tissues. All tests were two-sided, and P<0.05 was considered to indicate a statistically significant difference.

Results

ELISA. The KNG1 levels in the plasma, BALF and urine of patients with LUSC were significantly higher than those in benign controls (P<0.001) (Table II). The KNG1 level in BALF was significantly lower than that in plasma (P<0.0001), but significantly higher than that in urine (P<0.0001).

The OPN levels in the plasma and BALF of patients with LUSC were significantly higher than those in the controls (P<0.05 for both); however, the OPN level in urine tended to be lower in patients with LUSC than that in controls (P<0.05) (Table II). The OPN level in plasma was significantly higher than that in BALF and urine (P<0.001 for both), and there was also a significant difference in OPN level between BALF and urine (P<0.05). Notably, the OPN level in BALF was higher than that in urine in patients with LUSC (P<0.05), but lower than that in urine in the controls (P<0.05).

The AAT levels in the plasma, BALF and urine of patients with LUSC were significantly higher than those in the controls (P<0.01) (Table II). The AAT level in BALF was significantly lower than that in plasma (P<0.0001) but significantly higher than that in urine (P<0.0001).

Table II. Levels of OPN, AAT and KNG1 in the plasma, BAFL and urine of patients with LUSC and benign disease controls.

Marker	Unit	LUSC	Benign	P-value
KNG1				
Plasma	μg/ml	1,664.1±292.7	1,310.6±265.4	<0.0001
BALF	μg/ml	67.3±35.9	20.9±17.8	<0.0001
Urine	μg/ml	3.4±1.8	1.3±1.5	0.0010
OPN				
Plasma	ng/ml	4,8108.2±37,757.3	21,316.5±11,255.8	0.0107
BALF	ng/ml	160.3±223.0	32.7±47.1	0.0004
Urine	ng/ml	86.1±43.2	132.5±58.4	0.0088
AAT				
Plasma	μg/ml	25,082.7±9,145.2	16,589.1±9,138.7	0.0022
BALF	ng/ml	30,577.0±13,047.6	16,768.7±13,427.0	0.0014
Urine	ng/ml	2,176.9±1,536.9	788.2±690.0	0.0005

The proteins level in two groups were presented as mean ± SD. P-values were calculated by t-test (normally distributed continuous data) or Mann-Whiney U-test (non-normally distributed continuous data). P<0.05 was considered to indicate a statistically significant difference. LUSC, lung squamous cell carcinoma; KNG1, kininogen 1; OPN, osteopontin; AAT, α-1-antitrypsin.

ROC analysis. The AUC of the ROC curve, sensitivity and specificity values for KNG1, OPN and AAT in plasma, BALF and urine are shown in Table III. The results indicated that the combination of KNG1, OPN and AAT could improve the respective AUC values in plasma, BALF and urine (Table III).

KNG1, OPN and AAT protein expression in LUSC tissues. The results from IHC showed that KNG1, OPN, and AAT proteins were primarily expressed in the cytoplasm. The protein expression levels of KNG1, OPN and AAT were significantly increased in LUSC tissues compared with those in the controls (P<0.05) (Fig. 1, Table IV).

Discussion

Identification of biomarkers in various biological fluids is a promising strategy for lung cancer detection. Blood is the most studied biofluid with respect to biomarker discovery; thus, there is a wealth of information available regarding the blood proteome in multiple diseases. Considering its close association with lung tissue, BALF offers a means of assessing lung-related biomarkers, and previous BALF analysis results have shown some equivalence with biopsy findings (16). Urine is an ideal biofluid for biomarkers assessment owing to its ease of repeated noninvasive collection in large quantities. In addition, protein levels in urine tend to remain fairly stable due to low levels of proteolytic degradation.

The present study demonstrated the combined use of three biofluids (plasma, BALF, and urine) and tissues for lung cancer diagnosis purpose. To the best of our knowledge, this study is the first to describe the potential for KNG1, OPN and AAT to be used as diagnostic biomarkers of LUSC in plasma, BALF and urine specimens. The areas under the ROC curve of KNG1 in BALF was 0.91, and the AUC of KNG1 in urine was 0.81, which as good as in the plasma. The AUC of OPN in BALF was 0.83, while the AUC of OPN in urine was 0.75, which was better than that in plasma. The AUC of AAT in urine was 0.86, which was better than that in plasma and BALF. These results emphasized the potential of the above markers in urine and BALF to be used as diagnostic tools.

KNG1 protein is encoded by the KNG1 gene, and is a cysteine proteinase inhibitor that plays key roles in the process of blood coagulation. In addition, KNG1 has been found to play roles in cancer development, with different expression in different tumors. For example, KNG1 levels were decreased in the serum of patients with breast cancer (17), cervical cancer (18), and endometrial cancer (18), as well as in the urine of ovarian carcinoma (19) and renal cell carcinoma (20). Moreover, KNG1 was expressed at low levels in glioma cells (21) and renal cell carcinoma tissue (22). By contrast, increased KNG1 levels have been reported in the serum of patients with hepatocellular carcinoma (23), gastric carcinoma (24) and colorectal cancer (5). IHC staining revealed KNG1 expression to be significantly higher in colorectal cancer and advanced colorectal adenoma tissues than that in normal mucosa (25). Bioinformatics analyses suggested that KNG1 might play critical roles in colorectal cancer liver metastasis (26). KNG1 levels were also upregulated in other biofluids, such as the saliva of patients with oral squamous cell carcinoma (8) and the bile of patients with cholangiocarcinoma and pancreatic cancer (27). In our study, plasma, BALF and urine KNG1 levels were significantly higher in patients with LUSC compared with those in the controls, and this was also consistent with IHC data from 20 patients, who displayed higher KNG1 protein levels in tumor tissues than in normal tissues. We hypothesized that the observed increase in KNG1 levels in the assessed biofluids of patients with LUSC was a result of cancer cell-mediated production of this protein. While its specific mechanistic function in LUSC remains uncertain, KNG1 was thought to exert anti-angiogenic and anti-endothelial cell proliferative activities in certain cancers types (18). A previous study found that aberrant ceRNA-mediated regulation

Table III. Area under curve, sensitivity and specificity for KNG1, OPN and AAT in plasma, BALF and urine.

Marker	Cut-off level	AUC	Sensitivity, %	Specificity, %	P-value
KNG1					
Plasma	1,445.2 μg/ml	0.81	74	75	0.0002
BALF	30.0 μg/ml	0.91	92	73	<0.0001
Urine	1.3 μg/ml	0.81	90	59	0.0015
OPN					
Plasma	46,469.5 ng/ml	0.71	45	100	0.0115
BALF	40.9 ng/ml	0.83	81	93	0.0008
Urine	111.4 ng/ml	0.75	85	65	0.0105
AAT					
Plasma	20,393.7 μg/ml	0.74	65	75	0.0046
BALF	37,621.5 ng/ml	0.74	52	100	0.0020
Urine	628.4 ng/ml	0.86	100	53	0.0008

P<0.05 was considered to indicate a statistically significant difference. KNG1, kininogen 1; OPN, osteopontin; AAT, α-1-antitrypsin; AUC, area under the curve.

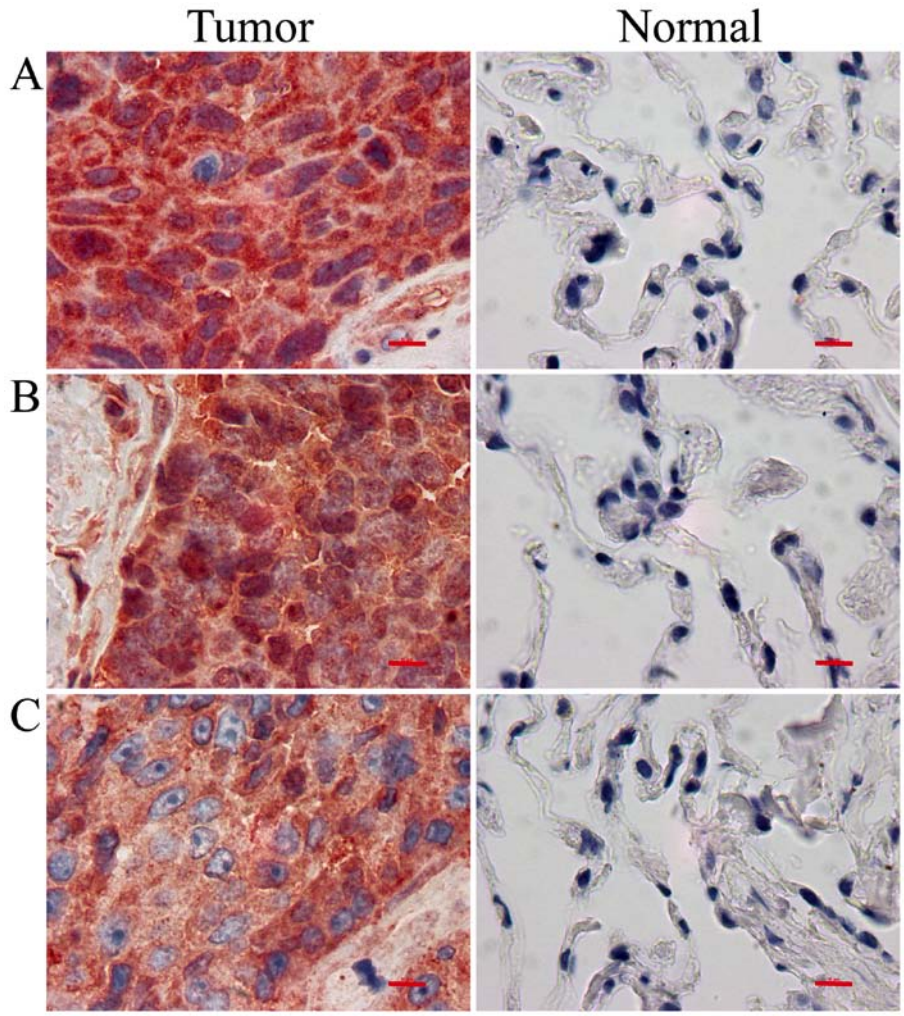

Figure 1. Expression levels of KNG1, OPN and AAT in LUSC and adjacent normal lung tissues were determined by immunohistochemistry. (A) KNG1 expression in LUSC tissues (left) and adjacent normal lung tissues (right). Positive expression of KNG1 was detected in LUSC tissue but not in the adjacent normal lung tissue. (B) OPN expression in LUSC tissues (left) and adjacent normal lung tissues (right). OPN expression was positive in LUSC tissue and negative in adjacent normal lung tissue. (C) AAT expression in LUSC tissues (left) and adjacent normal lung tissues (right). Positive expression of AAT was detected in LUSC tissue but not in the adjacent normal lung tissue. Scale bar, 10 μm; magnification, x1,000. LUSC, lung squamous cell carcinoma; BALF, bronchoalveolar lavage fluid; KNG1, kininogen 1; OPN, osteopontin; AAT, α-1-antitrypsin.

Table IV. Expression levels of KNG1, OPN and AAT in LUSC and adjacent normal lung tissues.

	LUSC tissue, n (n=20)		Normal lung tissue, n (n=20)		
Proteins	4-12	0-3	4-12	0-3	P-value
KNG1	9	11	2	18	0.031
OPN	8	12	1	19	0.020
AAT	7	13	1	19	0.044

LUSC, lung squamous cell carcinoma; KNG1, kininogen 1; OPN, osteopontin; AAT, α-1-antitrypsin.

of KNG1 contributed to glioblastoma-induced angiogenesis, which provided potential targets for the development of novel therapeutic strategies for glioblastoma (21). Importantly, KNG1 levels in plasma, BALF, and urine might serve as a potential LUSC biomarker, although the mechanism of KNG1 in LUSC needs further study.

OPN is a protein that plays crucial roles in immunity, remodeling of tissues and malignant transformation of tumor cells. The present study observed elevated OPN expression in the tumors of patients with LUSC, which was consistent with previous findings. Indeed, one study observed a significant link between OPN levels and gender, TNM stage, tumor differentiation, and poor outcomes in patients with NSCLC (28,29). OPN could play a variety of functions, binding with cluster of differentiation 44 or certain integrins to trigger the activation of the PI3K/AKT, Janus kinase 2, and focal adhesion kinase signaling pathways, thereby serving as a vital regulator of the epithelial-mesenchymal transition (30-32). Thus, OPN plays an essential role in cancer progression. OPN is a secreted protein that can be detected in different biofluids. High OPN levels in plasma were associated with higher levels of hypoxia in tumors and a higher risk of recurrence in patients with early stage NSCLC (33). In individuals with advanced lung cancer, higher circulating OPN levels were associated with a poorer prognosis and worse therapeutic responses (34-36). Prior to this study, BALF and urine OPN levels had not been assessed. Our findings revealed that OPN was upregulated in the plasma and BALF of patients with LUSC compared with those of healthy controls, but it was downregulated in the urine of patients with LUSC compared with that of healthy controls. In addition to tissue and blood, the levels of OPN in BALF and urine might also serve as a potential marker of lung squamous cell carcinoma.

AAT is a serine protease inhibitor that, while mainly produced by the liver, can also be found in other tissues and can be produced by cancer cells. Elevated AAT levels have been reported in patients with lung cancer (12,37), with higher plasma AAT levels being detected in these patients (14,38,39). AAT plays essential roles in the migration and invasion of cancer cells, regulating the assembly of fibronectin in the area surrounding a cell (13,40). The C-terminal portions of AAT were able to induce cell proliferation and invasion in human pancreatic adenocarcinoma (41), melanoma (42) and breast carcinoma (43). When AAT expression was reduced in a human or murine model, this led to a reduction in the observed proliferative, metastatic, and adhesive behavior of these tumor cells (37). AAT deficiency has been reported to increase the risk of lung cancer (44), and tumors positive for AAT had a poorer prognosis than those that were negative for this protein (45). In the present study, AAT protein levels were increased in LUSC tumor samples, suggesting that this protein might play a role in oncogenesis. Our results demonstrated that AAT levels in the plasma, BALF and urines of patients with LUSC differed from those in the normal controls. The AUC of AAT in urine was better than that in plasma and BALF, which suggested that detection of AAT in urine might be a non-invasive tool for LUSC screening.

Our findings suggest that the measurement of KNG1, OPN and AAT in body fluids, particularly in urine, represents a simple, non-invasive strategy that might be of value for LUSC diagnosis. To the best of our knowledge, this is the first study to simultaneously evaluate these three proteins in the plasma, urine and BALF of patients with LUSC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MZ and WW designed the study. WW and SW performed the experiments. WW wrote the article. SW and MZ reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The Ethics Committee of Beijing Shijitan Hospital, Capital Medical University approved the present study. All study participants provided written informed consent, and the study was performed following the Declaration of Helsinki.

Patient consent for publication

Patients provided written informed consent for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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