Receptor for advanced glycation end products (RAGE) soluble form (sRAGE): a new biomarker for lung cancer[1]

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Receptor for advanced glycation end products (RAGE) may be involved in the pathogenesis of cancer progression. Pathological effects mediated via RAGE are physiologically inhibited by soluble RAGE (sRAGE). The aim of this study was to identify sRAGE and RAGE expression profile in lung cancer patients. An ELISA method was used to quantify serum sRAGE in 45 individuals. Additionally, surgical specimens of 28 lung cancer patients were also included for RAGE expression by immunohistochemistry. Serum sRAGE was significantly decreased in lung cancer patients compared with controls (vs. healthy donors, P=0.034; vs. pulmonary tuberculosis patients, P=0.010). Lower sRAGE concentration was negative correlated with lymph node involvement (N0 vs. N1-2, P=0.028). Down regulation of membranous and cytoplasmic expression for RAGE was also lower in lung cancer tissue than in nearby normal lung tissue. Correlation with serum sRAGE concentration and RAGE expression in lung cancer tissue was existed by CV values. The results indicate that serum sRAGE levels are decreased during lung cancer progression and could reflect decreased RAGE expression in tissue. Serum sRAGE may serve as an effective and convenient diagnostic biomarker for lung cancer.

Key words: sRAGE, serum, RAGE, tissue, lung cancer

Lung cancer is the most common cancer in the world with nearly 1.35 million new cases per year, representing 12.4% of all new cancers. It has also been the most common cause of death from cancer with 1.18 million deaths [1]. Surgical resection is potentially curative treatment for lung cancer patients who are identified at an early stage. However, 30-60% of patients with stage I B to III A lung cancer die within 5 years after surgery, primarily from tumor recurrence [2]. Diagnosis and prognosis are determined based on tumor histological type, grade, size, lymph node involvement and several prognostic markers. Furthermore, all these methods are confined mainly to biopsies and it is also subjective depending on the pathologist's experience. Thus, it is required to find new molecular markers to effective and convenient diagnosis and predict the outcome of the disease.

Receptor for advanced glycation end products (RAGE) protein belongs to the immunoglobulin superfamily of cell surface molecules, which has initially been described as a receptor for advanced glycation end products (AGEs) [3–10]. AGEs are produced during the normal aging process and their production is accelerated in diabetes [11]. However, RAGE regulation has been documented in a large number of primary human carcinomas in recent years. In most cases, RAGE was up regulated in esophageal cancer [12], colorectal cancer [13] and oral squamous cell carcinoma [14], which acted as an oncoprotein. To our surprise, only in lung cancer, RAGE was down regulated and might behave as a tumor suppressor [15]. RAGE contains an extracellular domain (one "V"-type followed by two "C"-type), a single transmembrane spanning domain and a 43-amino acid cytosolic tail. V-domain of RAGE is, therefore, critical for ligand binding and the cytosolic tail is essential for RAGE mediated intracellular signaling. A secreted isoform of RAGE, termed soluble RAGE (sRAGE), has been identified as a novel splice variant carrying all of the extracellular domains but devoid of the transmembrane and intracytoplasmic domains. sRAGE was found to be released outside from cells, to bind the ligands of RAGE, and to be capable of preventing the

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[1]Abbreviations: AGEs – advanced glycation end products; CV – coefficient of variation, Ecs – endothelial cells; ELISA – enzymlibnked immunoabsorbent assay; ICAM-1 – intercellular adhesion molecule-1; VCAM – vascular adhesion molecule-1.

adverse effects of RAGE signaling [16]. In spite of its obvious relationship to cancer and metastasis, data focusing sRAGE deregulation in cancer is currently rare. Only in breast cancer was isentified that patients with better outcome (low grade and positive estrogen receptors) had higher sRAGE levels [17].

Given the potential correlation of sRAGE and RAGE, Nakamura K *et al.* found that sRAGE levels were significantly higher in type 2 diabetic patients than in nondiabetic subjects and positively associated with the presence of coronary artery disease in diabetes. The results showed that sRAGE level might be elevated in diabetes and could reflect enhanced RAGE expression in the diabetic vasculature [18]. Nakamura K *et al.* also demonstrated that Ang II up regulated RAGE mRNA levels in cultured micro-vascular endothelial cells (ECs) and subsequently increased sRAGE secretion into the medium of ECs [19]. However, to our best knowledge, no data concerning the relationship between tissue RAGE and serum sRAGE in lung cancer is available.

Here, in order to assess the possible role of sRAGE as a diagnostic and prognostic marker for lung cancer, we focused on preoperative serum sRAGE level by enzymelinked immunosorbent assay (ELISA) and RAGE expression by immunohistochemisty on lung cancer tissue, as well as their relationships with clinicopathological features. Further we estimated whether serum levels of sRAGE were correlated with tissue RAGE in lung cancer patients.

Materials and methods

Patients. A total of 73 individuals were enrolled in this study. The first set comprised 45 serum samples including 26 lung cancer (18 men, 8 women; aged from 52 to 80 years, mean: 68) and 19 controls (16 healthy donors, 3 pulmonary tuberculosis). The serum samples were collected via puncture of the cubital vein into tubes without anticoagulant after register. All samples were clotted for 30 minutes before centrifuged for 15 minutes at 1000×g, aliquoted and stored at -70 °C until used. Analysis of all samples was performed within 6 months after collection. Patients with diabetic, renal disease and some other elements that could affect serum sRAGE levels were excluded. The second set comprised 28 lung cancer patients (17 men, 11 women; aged from 35 to 78 years, mean: 60) which were also registered in Affiliated Hospital of Nantong University. All of the patients' tumor tissues and corresponding normal tumor adjacent tissues were obtained from resection specimens before receiving any other therapy.

Patients' evaluation before surgery included medical history, physical examination and blood tests. Chest CT scans had been routinely performed in patients of lung diseases. Location of the lung tumor was defined based on chest CT scan: peripheral when it is located in outer one third and central when it is located in inner two third in thoracic cavity. Pathologists had examined every dissected specimen and node during or after surgery. A sample of the primary tumor from each patient was fixed in 10% formalin and embedded in paraffin. Representa-

tive hematoxylin eosin stained sections were reviewed. For lung cancers, it included squamous cell carcinoma, adenocarcinoma, and adenosquamous carcinoma. Tumors were graded according to the World Health Organization grading system and were staged according to the TNM classification system [2]. The study was approved by local ethics committee.

sRAGE ELISA. Serum concentrations of sRAGE were measured with the Human sRAGE ELISA Kit (R&D Systems, USA) according to the manufacture's specifications. These samples were processed blindly. ELISA assay was run in duplicate. The range of the kits for sRAGE was 34.98-2418.90 pg/mL and the detection limit was 4.12 pg/mL.

Immunohistochemistry for RAGE. The Streptaridin-perosidase (SP) method was used to detect the RAGE protein following antigen retrieval with microwave treatment. After blocking endogenous peroxidase activity by incubating in 3% H_2O_2 for 10 min, specimens were rinsed with phosphate-buffered saline (PBS), and then incubated with antihuman RAGE antibody (polyclonal, 1:50; R&D, USA) at 4 °C overnight. Specimens were rinsed with PBS and incubated at room temperature for 30 min with secondary antibody; the samples were exposed to streptavidinperoxidase for another 30 min. After rinsing with PBS, diaminobenzidine (DAB) solution was used. Counterstaining was performed with haematoxylin. For negative controls only PBS was used.

Evaluation of immunohistochemistry was carried by Allred's score [20], which contained proportional scores and intensity scores. Proportional scores were classified into six grades, as follows: 0, no cell stained; 1, <1% cells stained; 2, 1%-10% cells stained; 3, 11%-33% cells stained; 4, 34%-66% cells stained; 5, all cells stained. Intensity scores were classified into four grades, as follows: 0, negative; 1, weak; 2, intermediate; 3, strong. We chose 20 microscopic fields at ×100 magnification and represented the mean of the summation of the proportional scores and intensity scores.

Statistical analysis. The SPSS 11.5 software package was used for statistical data analysis. Results of sRAGE levels were expressed as means±standard deviations. To compare results of serum sRAGE concentration in three groups, we used one-way ANOVA. Mean serum sRAGE levels and tissue RAGE expression for each variable, were compared using *t*-test or one-way ANOVA. The variability with the coefficient of variation (CV) statistic was used to analyze the relationship between serum sRAGE levels and tissue RAGE expression [21]. A *P*-value of<0.05 was regarded as statistically significant.

Results

Serum level of sRAGE. We evaluated the levels of sRAGE in serum obtained from lung cancer and pulmonary tuberculosis patients as well as healthy donors (Fig.1). Levels of sRAGE were significantly different (*P*=0.015) among the 3 study groups. The average level of serum sRAGE in healthy controls was 984.9±376.2 pg/mL. Patients diagnosed with lung cancer exhibited reduced levels of sRAGE (618.2±319.1 pg/mL),
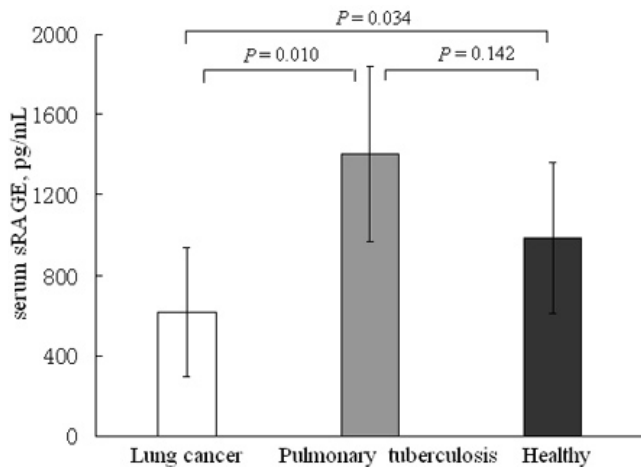
Figure1. Serum sRAGE levels in lung cancer and noncancerous patients. Serum was collected from lung cancers, pulmonary tuberculosis patients and healthy donors. Serum levels were determined using the sRAGE ELISA method, as described under the Materials and Methods section. The variance among the patients in the three groups was significant difference (*P*=0.015, one-way ANOVA). Lung cancer patients showed significantly lower sRAGE compared with pulmonary tuberculosis patients (*P*=0.010) and healthy controls (*P*=0.034). However, there was no statistical difference between pulmonary tuberculosis patients and healthy controls (*P*=0.142).
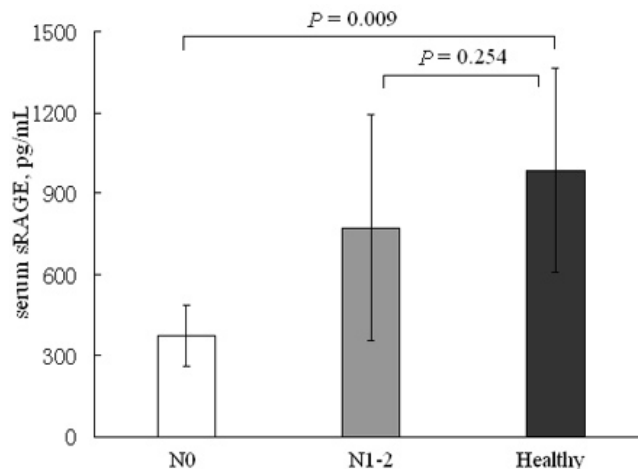

Figure2. Serum sRAGE levels in lung cancer patients of different lymph node involvement. Serum was collected from no lymph node involvement lung cancers (N0), N1-2 and healthy donors. N0 patients showed significantly lower sRAGE compared with healthy controls (*P*=0.009). However, there was no statistical difference between N1-2 and healthy controls (*P*=0.254).

Table1. Mean (standard deviation) serum sRAGE levels in patients with lung cancer broken down by clinicopathological parameters

Variables	Number of patients	Mean (S.D.,range) Serum sRAGE levels (pg/mL)	p-Value
Age			
<68.8 years	14	561.1(422.8, 171.8-1428.4)	
≥68.8 years	12	685.8(186.8, 275.5-947.4)	0.120
Sex			
Male	18	678.3(361.2, 171.8-1428.4)	
Female	8	484.4(212.3, 243.9-915.8)	0.209
Histologic subtype			
Adenocarcinoma	18	660.3(326.6, 171.8-1428.4)	
Non-adenocarcinoma	8	525.0(346.1, 171.8-1130.7)	0.776
Tumor size			
≤3 cm	16	718.9(200.3, 367.2-1130.7)	
>3 cm	10	458.9(460.4, 171.8-1428.4)	0.280
Differentiation			
Moderate	16	487.4(325.1, 171.8-1428.4)	
Poor	10	830.1(240.9, 275.5-1130.7)	0.780
Lymph node involvement			
N0	10	374.7(45.5, 243.9-571.6)	
N1-2	16	771.5(362.7, 171.8-1428.4)	0.028
Pathologic tumor factor(pT)			
T1	14	745.9(210.8, 367.2-1130.7)	
T2-4	12	469.4(403.2, 171.8-1428.4)	0.527

significantly lower than healthy control values (*P*=0.034). Furthermore, a substantial increase in sRAGE levels was identified in pulmonary tuberculosis patients (1404.3±436.3 pg/mL), which was higher than in lung cancer group (*P*=0.010). However, paired comparison of the various groups revealed that pulmonary tuberculosis patients did not significantly differ in serum sRAGE levels from healthy donors.

These results indicated that elevation of sRAGE levels was common in healthy donors and pulmonary tuberculosis patients, the latter being more prominent while in lung cancer sRAGE was reduced.

Relationship between serum sRAGE levels in lung cancers and clinical and pathological features of the individuals. To further characterize sRAGE levels in lung cancer patients, the associations of serum sRAGE levels with various clinical and pathologic variables were presented in Table 1. A three-fold decrease of serum sRAGE levels was observed in lung cancer patients with no lymph node involvement (N0, 374.7±45.5 pg/mL) and a low amounts of sRAGE were already detected in lung cancer patients with N1-2 (771.5±362.7 pg/mL), differences that were statistically significant (N0 vs. N1-2, *P*=0.028). But we found that sRAGE levels were not correlated with age, sex, histology, tumor size, tumor differentiation and pathologic tumor factor.

These findings showed that serum sRAGE levels were negative correlated with lymph node involvement in lung cancer patients. Furthermore, we found statistical difference was especially predominant between N0 and healthy donors (N0 vs. Healthy, *P*=0.009) (Fig.2).

Immunohistochemistry for RAGE. Because decreased levels of sRAGE were detected in serum of lung cancer patients,
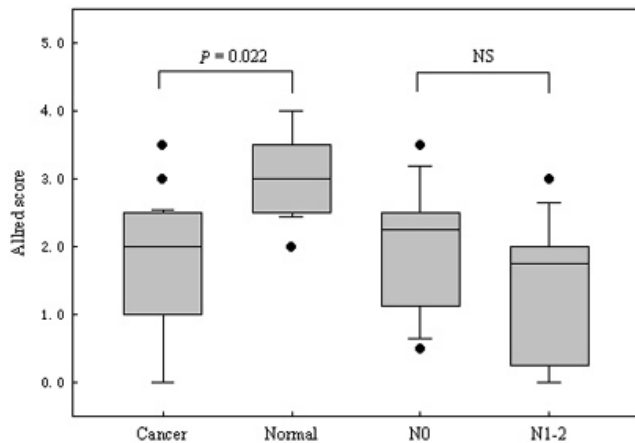
Figure3. Tissue RAGE expression in lung cancer and corresponding normal tumor adjacent tissue. RAGE expression was detected by immunohistochemistry and evaluation was carried by Allred's score. Mean RAGE expression (±SD) were represented by the box; medians are plotted as a line inside the box; error bars represented 10th and 90th percentiles; dots outside box were values that falls outside the 10th and 90th percentiles. Lung cancer tissue RAGE expression was lower than that of corresponding normal tissue (P=0.022) (A). There was no apparent correlation in RAGE expression between N0 group and N1-2 group (B).

immunhistochemical staining was used for detecting tissue RAGE expression in lung cancer patients. We studied expressions of RAGE in lung cancer tissue and corresponding normal tumor adjacent tissue. Cells showed membranous and cytoplasmic expression for RAGE. The Allred's score of RAGE expression was 1.64±0.94 in lung cancer tissues, which was significantly lower than that in corresponding normal tissues 3.07±0.61 (P=0.022). However, in tissue RAGE expression, there was no correlation between the RAGE levels and lymph node involvement (Figs. 3 and 4).

The above results demonstrated that in tissue, RAGE also showed the decreased expression with lung cancer.

Relationship between serum sRAGE concentration and RAGE expression in lung cancer tissue. For the both of low sRAGE and RAGE in lung cancer patients, we used CV value to examine the possible relationship between sRAGE and RAGE. The concentration of serum sRAGE was 618.2±319.1 pg/mL in lung cancer patients. The CV of sRAGE concentration in lung cancer patients' serum was 62.63%. Furthermore by the Allred's score, a score of RAGE expression in lung cancer tissues was 1.64±0.94. The CV value was 57.32% of RAGE expression in lung cancer tissue. The CV of serum sRAGE concentration closed to the CV of RAGE expression in lung cancer tissue (Fig. 5).
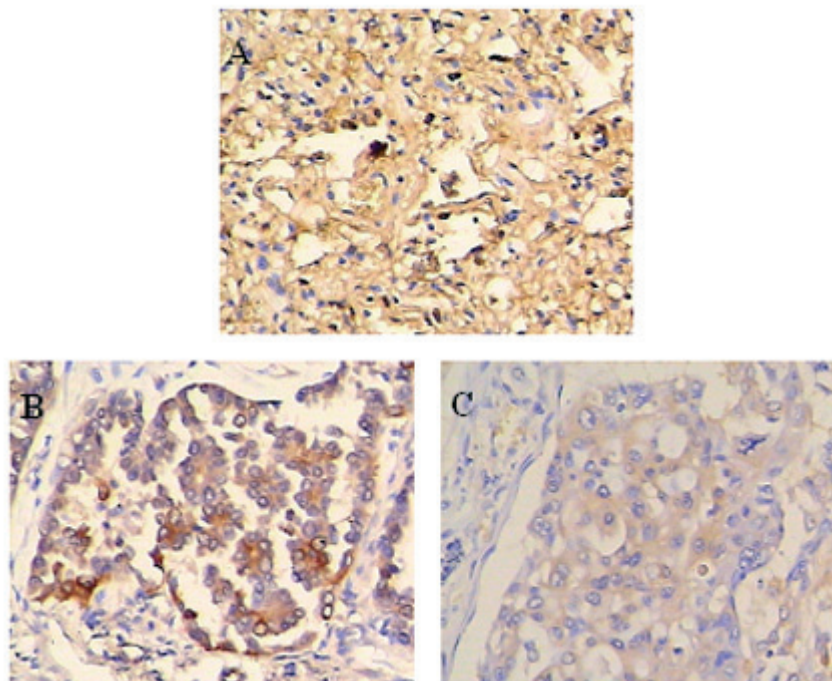

Figure4. Immunohistochemical stain of RAGE. The cytoplasm was stained strongly in lung normal tissue, but almost no cytoplasm was stained in lung cancer tissue (×400). High expression of RAGE was observed in normal tissue (A) than in lung cancer tissue with N0 (B) and lung cancer with N1 (C).
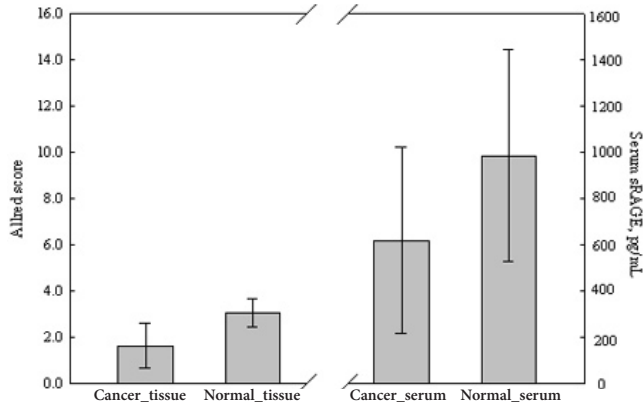
Figure 5. Serum sRAGE reflected tissue RAGE expression of lung cancer patients. Both the RAGE in tissue and sRAGE in serum of lung cancer patients were decreased trend. The CV of sRAGE in lung cancer patients' serum was 62.63% which was closed to the CV (57.32%) of RAGE expression in lung cancer tissues.

These results suggested that serum sRAGE might reflect RAGE expression in lung cancer tissue.

Discussion

RAGE up regulation was observed in essentially most primary human tumors examined [15]. However, the adult lung is unique in that it expresses high amounts of RAGE under normal conditions while other tissues express low amounts normally, and its soluble form (sRAGE) acts as a decoy receptor. Both of them may play a novel and homeostatic role in lung physiology [22], thus positioning sRAGE as an effective and convenient biomarker in lung cancer patients is possible. Currently, rare study can be found in examining serum sRAGE and its diagnostic significance in lung cancer [23]. Here, we provided evidence that serum sRAGE levels correlated with lung cancer progression. Importantly, decreased serum sRAGE was found in lung cancer patients and lower concentration serum sRAGE was associated with lymph node involvement. By immunhistochemical staining, we also identified that in lung cancer tissue there was lower expression of RAGE than in normal tissue. No correlation was found between RAGE levels and lymph node involvement in lung cancer tissue. sRAGE is a variant of RAGE, thus, it appears that sRAGE' production is correlated with RAGE expression in tissue. To further verify this aspect, we used the CV value of sRAGE and RAGE to analyze correlation and compare by method was referenced by Kumura [21]. sRAGE level in lung cancer patients' serum could reflect decreased RAGE expression in the lung cancer tissues.

Several factors may account for the down regulation of serum sRAGE in lung cancer patients. Circulating sRAGE may prevent the different RAGE ligands from binding to cellular RAGE receptors. The role of AGE and other RAGE ligands such as amphoterin [24] and S100 [25] proteins with the potential relation to the invasion and the formation of metastases may be important. Binding of AGEs or other ligands to RAGE results in the activation of the key mediators of the proliferation such as p21ras, mitogen-activated protein (MAP) kinases, nuclear factor (NF)-κB, and cdc42/rac, which lead to their downstream effects (stimulation of cell proliferation, invasion, and metastases). Taguchi *et al.* found that blockade of the amphoterin/RAGE system (by the suppression of p44/p42, p38, and SAP/JNK MAP protein kinase) in mice suppressed the migration and invasivity of tumor cells and possibly also cell proliferation and the production of tissue metalloproteinases [26]. Increased sRAGE could interact with different RAGE ligands (AGEs, amphoterin, S100 proteins) and impair RAGE mediated stimulation for tumor growth and invasion. The above findings suggest a protective role of sRAGE, but the mechanisms are quite complicated and there are many questions to be answered.

In addition by examining sRAGE concentration in lung cancer, this study also characterized the association of sRAGE with clinical and pathological features. Lower concentration serum sRAGE was associated with lymph node involvement. sRAGE may be a diagnostic indicator for detecting lung cancer patients' lymph node involvement. Although a similar result was found in breast cancer patients [17], we were not able to confirm the higher levels of sRAGE in lung cancer patients with N1-2 than N0, possibly due to the under representation of those no lymph node involvement of lung cancer in this group of newly diagnosed patients.

Recent studies have shown that RAGE is expressed in variety of neoplastic cell types and has been associated with cell proliferation, apoptosis, adhesiveness, migration and invasiveness [27]. Expression pattern of RAGE was altered in many types of cancers. Therefore, several attempts to use RAGE expression as diagnostic indicator are under development. Sasahira *et al.* showed that RAGE expression was significantly higher in adenomas and associated with severe atypical and tumor' size. Another report demonstrated that high expression of RAGE in oral squamous cell carcinoma was associated with depth of invasion and local recurrence [14]. Disease-free survival in patients with high-grade expression of RAGE (RAGE-H) was significantly worse than in those with low level RAGE expression. To further assess the expression of RAGE in lung tissue, immunhistochemical staining was explored . Decreased expression of RAGE was found only in lung cancer tissue. The results were consistent with these of Bartling *et al.* [15]. Our previous study thought that RAGE could be associated with either enhancing or inhibiting growth depended on tissue types [12]. In lung, developmental expression of the RAGE in the neonatal rat was reported by Lizotte *et al.* [22]. It reported that rat pulmonary RAGE expression increased with age beginning from birth and played a novel and homeostatic role in lung physiology. We supposed specific behavior of RAGE expression in lung tissue.

Both serum sRAGE and tissue RAGE expression was decreased in lung cancer patients, we compared the change of CV value for different evaluate criterion and measuring units [21]. The CV of serum sRAGE concentration closed to the CV of RAGE expression in lung cancer tissue. We supposed that serum sRAGE was related to tissue RAGE expression in lung cancer, which was consistent with recent findings. Steiner *et al.* found elevated serum levels of soluble forms of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in patients with diabetes reflected upregulation of ICAM-1 and VCAM-1 in ECs. RAGE belongs to the same immunoglobulin superfamily as ICAM-1 and VCAM-1 [28]. Pachydaki *et al.* also found that vitreous levels of sRAGE were increased in proliferative retinal diseases by reflecting enhanced RAGE expression in epiretinal membranes of the eyes [29]. However, the correlation with serum sRAGE and tissue RAGE expression in lung cancer patients still need further investigation.

In conclusion, our results support the previous study that deregulation RAGE may be a possible diagnostic indicator for cancer. Our study also identify that lower serum sRAGE concentration is correlated with lymph node involvement in lung cancer and could reflect decreased tissue RAGE expression. Serum sRAGE may serve as an effective and convenient diagnostic biomarker for lung cancer especially for detecting lymph node involvement.

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